ABSTRACT

Title of Document:	A STUDY OF PH MANIPULATION ON TUMOR PROLIFERATION AND THE CTL RESPONSE
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Adoptive Cell Transfer (ACT) Therapy is a cancer treatment that enhances and utilizes the body's own immune system. However, this treatment has had limited success in clinical trials. We hypothesized that this is due to the immunosuppressive, acidic microenvironment of cancer tumors. We tested the effects of acidic, neutral, and basic environments *in vitro* on cytotoxic T lymphocyte (CTL) survival, activation, migration and killing ability and on cancer cell survival. We found that CTLs have most optimum survival, activation, and migration in a neutral environment, while the optimal extracellular conditions for EG-7 lymphoma are slightly acidic and B16-OVA melanoma survives best in physiological conditions. Future research should further study the killing ability of T cells in the three different environments and look to move to *in vivo* experiments.

A STUDY OF PH MANIPULATION ON TUMOR PROLIFERATION AND THE CTL RESPONSE

By

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Chapter 1: Introduction

Human Immune System

The human immune system is a complex defense network within the body that protects the body from infection by foreign substances and organisms. From the initial site of infection or entry to the eradication of a pathogen, the immune system protects the body through many different mechanisms and pathways. The attack system works at two levels of immunity – a primary, immediate, and innate immunity; and a secondary, specific, and memory-inducing adaptive immunity (Adam, Odhav, & Bhoola, 2003; Post-White, 1996). Both of these subsystems are composed of multiple cells types and proteins that work in conjunction to communicate and destroy the "non-self" invaders.

The innate response, present since birth, is the primary, general response that occurs when the foreign substance first enters the body. It recruits more immune cells to the site of infection by releasing cytokines. The innate response also activates the adaptive immune system through multiple means, one of which is antigen presentation.

The adaptive response is the more complex, secondary response. It can recognize a wide range of foreign substances and retains memory cells that can quickly respond to subsequent challenges by the same substances (Adam et al., 2003; Post-White, 1996). A crucial component of the adaptive response is the *major histocompatibility complex* (MHC), a protein on the surface of all cells that serves as an identification molecule. This molecule can be recognized by the adaptive immune system as "self" or "non-self" by presenting a segment of a protein within the cell, or an antigen, on the cell surface. Selection during immune cell development is what allows immune cells to distinguish between antigens from normal cells or antigens that have previously

been un-encountered. As T cells mature in the thymus, most of those that are self-reactive, or recognize and kill "self"-antigen presenting cells, are eliminated. Those that are not eliminated may undergo receptor editing, where reactive T cell receptors are replaced by nonreactive receptors. Other remaining self-reactive cells may lose their functional capabilities upon further exposure to a "self" antigen (Sakaguchi et al., 2008). The vast majority of cells that complete this process do not kill normal, healthy, "self"-antigen presenting cells and can kill many previously un-encountered antigens.

A previously un-encountered antigen can originate from a foreign pathogen infecting the cell or an altered cell producing foreign proteins, such as certain cancer cells. There are two main types of adaptive immunity: humoral and cell-mediated. We focus primarily on cell-mediated immunity because it is the part of the immune system that primarily fights off cancer cells, a focus of our research.

Cell-mediated immunity involves mostly T lymphocytes, or T cells. T cells include helper T cells (CD4+ T cells), which enhance the functions of multiple cells in the immune system, and cytotoxic T cells (CD8+ T cells), which are primarily involved in destroying virus-infected cells or tumor cells. In order for T cells to recognize "non-self" antigens, cells must have Major Histocompatibility Complex (MHC) proteins present on their cell surfaces because the MHC binds and presents the antigen to T cells. In addition to possessing the ability to recognize harmful cells and substances, a CD8+ T cell must be activated to kill off infected cells. Activation results in a cytotoxic T lymphocyte (CTL) that is able to recognize one type of antigen and is also able to kill cells presenting that specific antigen (Verlingue et al., 2014; Rescigno, Avogadri, & Curigliano, 2007; Post-White, 1996; Restifo et al., 2012; Yee, 2005; Dermime, Armstrong, Hawkins, & Stern, 2002). Despite the specificity and complexity of the

immune system, some diseases are still able to evade this defense mechanism. One of the most notable groups of diseases that the immune system struggles to overcome is cancer.

Cancer and the Immune System

Cancer begins with a genetic mutation that can occur in one of any number of genes. Further mutations of specific genes in a few cells of the body can then lead to uncontrollable cell growth (Lerman & Shields, 2004). This growth is supported by a process known as angiogenesis, the formation of new blood vessels, which are used to nourish the tumor (Hanahan & Folkman, 1996). The close proximity of these blood vessels also allows the tumor to metastasize, or spread, to other locations in the body (Hanahan & Weinberg, 2011; Hollstein et al., 1991). Metastasis is especially dangerous because many commonly used treatments cannot reach areas of metastasis and multiple organ systems can then affected by the cancer.

Conventional treatments, such as chemotherapy, and radiation, do not specifically target cancer cells in the body, and as a result often harm other body cells (Heinemann, Douillard, Ducreux, & Peters, 2013). Surgery removes tumor or cancerous tissue from the body, but can leave some undetected cancerous cells or metastasized lesions behind. For certain cancers, chemotherapy or radiation often follows surgery in an attempt to kill any remaining cancerous cells. Chemotherapy involves the systemic administration of a drug or multiple drugs that kill quickly proliferating cells, a characteristic that is not exclusive to cancer cells. Several cell types in the body that normally proliferate rapidly, including immune cells, hair follicles, bone marrow, and cells within the digestive tract, can be compromised during chemotherapy (Mitchison, 2012). Radiation therapy exposes cancerous cells to X-rays, gamma rays, and charged particles; and kills the cells by damaging the DNA beyond repair (Lawrence, Ten Haken, & Giaccia, 2008). However, radiation itself, by damaging and mutating DNA, is a

carcinogen and can cause secondary cancers to develop (Sountoulides, Koletsas, Kikidakis, Paschalidis, & Sofikitis, 2010). These treatments, with their debilitating side effects, have become standards in medicine because the body alone cannot fight cancer in the same way it can fight foreign infection. The body's immune system could have difficulties identifying and killing cancerous cells because cancers have a variety of mechanisms that suppress or direct the immune system away from malignant cells.

Immunosuppressive Nature of Cancer

Immunosuppression refers to the weakening and subsequent decrease in efficiency of the immune system, and is caused by tumor formation and development (Baruch, 2005). The immunosuppressive nature of tumors and associated cancer cells allow them to largely avoid detection and killing by the immune system.

The immunosuppressive nature of tumors is partially caused by recruitment of immature myeloid cells (ImCs) at the site of the tumor (Kusmartsev & Gabrilovich, 2002; Motz & Coukos, 2011). Physiologically, one of ImCs potential functions is to prevent autoimmune actions of T cells. Secretion and production of reactive oxygen species by immature myeloid cells as well as factors such as vascular endothelial growth factor (VEGF), IL-10, transforming growth factor (TGFβ) from the tumor cells suppress IFN- ɣ production. Because IFN- ɣ production plays a critical role in CD4+ and CD8+ T cells' function in the immune system, antitumor immunity is compromised. VEGF is a downstream target of HIF-1, and is thus activated by hypoxic factors (Ankoma-Sey, Wang, & Dai, 2000; Forsythe et al., 1996; Ziello, Jovin, & Huang, 2007). VEGF leads to vascularization of the tumor environment, while also promoting the immunosuppressive nature of cancer. Thus, hypoxia indirectly leads to the evasion of cancer cells from antitumor immunity (Noman et al., 2011; Noman et al, 2015; Sitkovsky, 2008).

Another defense mechanism is that tumor cells are also able to adopt the phenotypic characteristics of lymphoid cells, cells of the innate immune system. This immunosuppressive characteristic enables tumor cells to migrate using the same mechanisms as lymphoid cells, allowing them to penetrate the endothelium and acquire mobility within tissues and blood vessels to metastasize. However, circulating tumor cells are particularly sensitive to lysis by natural killer cells or monocytes. Thus, if the immune system of an individual is strong, it may be able to destroy the migrating tumor cells (Arias et al., 2011).

Immunoediting, a process by which the immune system itself alters tumorigenesis, further exacerbates the problem of unrecognizable cancer cells. T cells kill tumor cells that they recognize. Over time, cancer cells that evade detection and killing by the immune system grow to be a greater proportion of cells in the tumor. Eventually, very few of the remaining tumor cells are recognizable by the immune system (Dunn et al., 2004, Mittal et al., 2014, Restifo et al., 2012; Schreiber, Old, & Smyth, 2011).

There are multiple ways that those cancerous cells became unrecognizable. Tumor cells can be rendered unrecognizable either by the loss of tumor antigen expression, which can occur with the loss of MHC class I proteins on the tumor, or the loss of antigen processing functions within the tumor. These processes, in addition to recruitment of ImCs, eventually causes the tumor to become invisible to the immune system, allowing it to continue growing (Schreiber et al., 2011). The phenomenon of immunoediting suggests that immune cells can both prevent tumor formation, by killing nascent tumor cells, and promote tumor progression, by selecting for tumor cells that can evade immune detection and killing. (Schreiber et al., 2011).

These are some of the immunosuppressive characteristics of tumors. Another immunosuppressive characteristic of cancerous tumors, the acidic microenvironment, is a focus of our research.

The Acidic Microenvironment of Cancerous Tumors

Cancerous cells have an abnormal metabolism to accommodate the high-energy they require (Gillies, Raghunand, Karczmar, & Bhujwalla, 2002; Swietach, Vaughan-Jones, Harris, & Hulikova, 2014; Warburg, Wind, & Negelein, 1927). Typically only cells in an environment without oxygen produce energy through glycolysis; cells in aerobic condition primarily produce energy through cellular respiration. In the 1920s, German researcher Otto Warburg observed that tumor tissues in aerobic conditions performed glycolysis ten times more frequently than normal tissues (De Milito et al., 2010; Gillies et al., 2002; Kato et al., 2013; Swietach et al., 2014; Tannock & Rotin, 1989; Warburg et al., 1927). He also observed that this high rate of glycolysis and cellular respiration occurred simultaneously (Warburg et al., 1927). Together, glycolysis and cellular respiration produce more energy than respiration alone. This abnormal metabolic characteristic of cancer tumors is known as the Warburg Effect.

One result of glycolysis is the production of lactic acid, which has a pKa of 3.9 at pH 7.4 (Phypers & Pierce 2006). At physiological pH, lactic acid, dissociates to lactate and a proton (Phypers & Pierce 2006, Swietach et al., 2014). For each glucose molecule that undergoes glycolysis, two protons are produced. Because glycolysis occurs in cancer cells at a rate that is about 200 times higher than that in normal cells, a very large concentration of protons are produced and accumulated within the cell, creating an acidic environment (Warburg 1956). The cell cytosol is sensitive to pH changes and thus, the cell uses proton pumps to send large amounts

of protons out of the cell into the cellular microenvironment. This mechanism maintains the internal pH of the cell and creates an acidic microenvironment. There is evidence, as discussed later in the literature review, that immune cells either die or become deactivated in an acidic environment (Lardner, 2001; Kato et al., 2013; Muller, Fischer, & Kreutz, 2000; Restifo et al., 2012). It should be noted that immune cells also exhibit the Warburg effect and similar metabolism to cancerous cells (Palsson-McDermott & O'Neill 2013).

Immunotherapy

Immunotherapy is part of the movement toward cancer treatments that target specific characteristics of cancerous tumors or the immune cells fighting the cancer (Hinemann et al., 2013; Riethdorf & Pantel, 2010; Rosenberg, Restifo, Yang, Morgan, & Dudley, 2008; Verlingue et al., 2014). Immunotherapy involves improving the immune system's ability to fight cancer. There are many types of cancer immunotherapy, including antibody therapy, cytokine and anticytokine therapy, vaccination, oncolytic virus therapy, and adoptive cell transfer therapy (ACT) (Zhou 2014). Antibody therapy involves using antibodies that can bind to cancer antigens in order to alter the immune system or deliver drugs to cells presenting cancer antigens (Scott et al., 2012). Cytokine therapy involves using cytokines, proteins that allow immune cells to communicate with each other, to stimulate effector and stromal cells as well as promote cancer recognition by cytotoxic T lymphocytes (Lee & Margolin 2011). Interleukin 2 (IL-2) and interferon alpha (IFN- α) cytokine therapies have been approved to treat certain cases of melanoma (Lee & Margolin 2011). Anticytokine therapy involves blocking certain cytokines that can promote tumor development. It has mainly been researched for use with autoimmune

diseases but one recent study has indicated that reducing the action of Interleukin-6 (IL-6) could reduce colorectal cancer tumorigenesis (Wang et al., 2013, Zhou 2014).

Another promising form of immunotherapy, vaccination, consists of injecting proteins, portions of proteins known as peptides, dead cancerous cells, altered white blood cells, or other biological compounds designed to help the immune system recognize cancer and destroy it. One vaccine, Sipuleucel-T, is approved by the U.S. Food and Drug Administration (FDA) for use in patients with metastatic prostate cancer (Schlom 2012). In addition, oncolytic virus therapy is an emerging type of immunotherapy. In October of 2015, Imlygic became the first oncolytic virus therapy approved by the FDA. Treatment with Imlygic consists of injecting malignant melanoma lesions with a modified herpes virus that replicates inside cancer cells, destroying them by causing them to burst (U.S. Food and Drug Administration, 2015).

Finally, Adoptive Cell Transfer (ACT) Therapy is a cancer immunotherapy that enhances a patient's immune system outside of the body (Hawkins et al., 2010; Restifo et al., 2012; Yee et al., 2002; Yee, 2005). This form of immunotherapy is the model used in this project.

Adoptive Cell Transfer (ACT) Therapy

Adoptive Cell Transfer (ACT) Therapy is a cancer immunotherapy that enhances a patient's immune system outside of the body (Hawkins et al., 2010; Restifo et al., 2012; Yee et al., 2002; Yee, 2005). ACT can take on two forms. One form multiplies and enhances the killing ability of T cells that are already predisposed to targeting the tumor, known as tumor infiltrating lymphocytes (TILs). These TILs are purified from a biopsy of the tumor, enhanced with IL-2, multiplied *in vitro*, and then returned to the patient (Mondino et al., 2010; Hawkins et al., 2010; June, 2007; Restifo et al., 2012; Rosenberg et al., 2008). Another form of ACT uses circulating T cells isolated from a patient's blood. Since these T cells do not specifically recognize the cancer,

they are then genetically modified with specific receptors that will recognize the tumor (Perica, Varela, Oelke, & Schneck, 2015; Rosenberg et al., 2008; Yee et al., 2002). In both forms of ACT, the T cells are extracted, activated with cytokines, given time to multiply and expand, and reintroduced into the patient's body (Mordino et al., 2010; June, 2007; Rosenberg et al., 2008; Yee et al., 2002).

Prior to reintroducing the T cells into the body, the patient goes through immune-depletion, the process of administering a chemotherapeutic drug or total body irradiation to kill the majority of the patient's viable immune cells throughout the body (Perica et al., 2015; June, 2007; Rescigno et al., 2007; Restifo et al., 2012; Rosenberg et al., 2008). The new T cells are gradually reintroduced to the patient, so the body can adapt over time. IL-2 is also administered to the patients to aid the expansion of the T cells *in vivo* (Hawkins et al., 2010; Restifo et al., 2012; Rosenberg et al., 2008). ACT is considered an advantageous treatment because it utilizes immune cells with specificity for the cancer. Additionally, the cells in question are from the patient's own body, minimizing the chance of the treatment being rejected (Mazzarella et al., 2012). However, treatment with ACT can be a lengthy, and costly process, as a large volume of CTLs would need to be cultured: for patients with aggressive cancers, this is time that they may not have.

Immune cells that are impeded by an acidic microenvironment may result in less effective immunotherapy treatments that are meant to enhance the immune system's ability to differentiate cancer cells from body cells and, thereby, kill cancer cells more efficiently (Ely, 2009; Friedmann-Morvinski & Eshhar, 2006; Hawkins et al., 2010; Restifo, Dudley, & Rosenberg, 2012).

Our Approach

We were particularly interested in the potentially immunosuppressive nature of the acidic tumor microenvironment, and its effect on immune cell function and killing ability. Given that tumors exhibit an acidic microenvironment and such environments inhibit T cell recruitment and function, manipulating this environment to a physiological pH could potentially improve the efficacy of ACT by allowing more reintroduced immune cells to reach the tumor and preventing them from being killed or deactivated. If manipulating the pH of the tumor microenvironment allows stronger immune cell function, then potentially fewer immune cells would be needed in ACT, reducing the cost and time necessary for the treatment.

Research Question

What are the effects of pH manipulation on tumor proliferation and the cytotoxic T lymphocyte response?

Hypothesis

We hypothesized that manipulating the acidic tumor microenvironment toward a physiological pH would inhibit tumor proliferation and enhance T lymphocyte activation and killing ability.

Research Strategy

This study consisted of three phases, or aims, each of which took place *in vitro*. It was necessary to first observe the effects of pH adjustment on cancer cells and immune cells individually. To clarify, pH manipulation refers to the external cellular pH of the cell growth media, not the internal pH of the cell.

The first aim of this study was to determine the effect of pH manipulation on cancer cell survival. Two types of cancer cells, EG7 lymphoma and B16-OVA melanoma, were used during this phase of the experiment to understand whether different cancer types had different behavior in acidic, neutral, and basic pH environments. These specific cancer models were chosen because both types of cancer have been used to study immunotherapy previously (Rosenberg et al., 2008). A secondary pH shock experiment was also set up to determine if differences in cell growth were due to short-intense periods of pH change or sustained pH differences. Data collected was total alive cell counts for each group.

The second aim of this study was to determine the effect of pH manipulation on immune cell survival, activation, and migration ability. Studying the survival of immune cells independently allowed for the observation of non-cancerous cells in acidic, neutral, and basic pH environments. Migration assays were conducted to examine how the migration of immune cells was impacted by the pH of the environment. Comparing the activation and migration ability after culture in different pH environments determined the impact of pH on the function of immune cells.

The third aim of this study was to simulate ACT *in vitro,* with melanoma cells and activated immune cells cultured in the same dish in various pH environments. Killing assays were performed to determine the percent killing for each combination of melanoma cells and activated immune cells.

Naturally, *in vivo,* CTLs are generated from naïve T cells through activation, a three signal process that triggers proliferation, differentiation, and cytokine secretion (Mescher et al., 2006). The first activation signal, Signal 1, occurs when a T cell receptor interacts with an antigenic peptide-bound MHC molecule on an antigen-presenting cell (APC) (Lafferty, Prowse,

Simeonovic, & Warren, 1983). Signal 2, costimulation, occurs when B7, a costimulatory molecule present on the surface of activated APCs, engages with CD28, a costimulatory protein expressed on surface of T cells (Lafferty et al.,1983; Jenkins et al., 1991; Lenschow et al. 1996). Signal 2 magnifies Signal 1 and together, these two signals trigger clonal expansion. This expansion is amplified by Interleukin 2 (IL-2), the production of which increases in response to antigenic stimulation and costimulation (June et al., 1987; Lanzavecchia & Sallusto, 2001).

The third required signal is inflammatory and signals to T cells that there is a threat in need of a response. Signal 3 is also important for survival, differentiation, effector function, and the development of memory T cells. For naïve CD8+ T cells, this signal can be provided by Interleukin 12 (IL-12) and type I interferons (Curtsinger et al., 1999; Valenzuela et al., 2002). Once activated, antigen-specific CTLs release a variety of molecules, including IFN-γ, a cytokine that regulates pathogen recognition and inhibits proliferation of target cells, and Granzyme B, a protease that kills pathogens and cancer cells.

The *in vitro a*ctivation of naïve T cells to generate CTLs is similar to the aforementioned process observed *in vivo*. We used OT1 CD8+ T cells. OT1 cells express a T cell receptor that recognizes MHC Class I peptides when they are bound to SIINFEKL, a short amino acid sequence from chicken ovalbumin protein (Ma et al., 2009). The isolated OT1 cells were plated in a dish coated with Dimer X, a recombinant MHC Class 1, then B7 was added as a costimulator. By pulsing the plate with SIINFEKL, signals 1 and 2 were provided, bringing about proliferation. To provide the third signal, IL-12 was introduced and subsequently improves the killing ability of the CTL.

Chapter 2: Literature Review

As previously stated, ACT is a promising treatment as it utilizes the body's own immune cells to fight cancer; however, ACT could be improved by improving the immune cells' ability to migrate to, function, and kill at tumor sites. Though Adoptive Cell Transfer therapy has been studied in the past, there are few studies that examine ACT in combination with pH manipulation. Our approach will examine ACT in melanoma and the effects of various pH levels on the killing ability of T lymphocytes.

Successes and Drawbacks of ACT

Melanoma is the cancer most commonly studied for ACT because it has been shown to be one of the most effective treatments for patients with this cancer (Rosenberg et al., 2008). This treatment is successful because it is relatively easy to isolate tumor-specific T lymphocytes that are found surrounding melanoma tumors (Hawkins et al., 2010).

Unfortunately, this method has only had modest success, when used as the only treatment. In addition to having a weaker effect on cancer than desired, ACT requires a large amount of cells for transfer to be effective (Yee, 2005; June, 2007). While many T cells must be cultured from biopsied tumors, only a little over one-third of biopsied portions produce viable T cell populations of adequate quantity. Furthermore, the process from biopsy to re-infusion requires an intense amount of time, funds and labor (June, 2007). Lastly, the cells enhanced and expanded *in vitro* have limited lifespans (Yee, 2005).

These inefficiencies of treatment also make clinical trials difficult to perform (June, 2007). Finding a way to use fewer T-cells in treatment is thus a promising avenue to make research and treatment with ACT more practical.

One avenue that has been explored in using fewer T cells is coupling ACT with T Cell Receptor (TCR) gene therapy. In this system, the T cells are genetically programmed to express a receptor specific to cancer-testis (CT) antigens, which are predominantly expressed in germ cells and certain malignancies. This allows for a more targeted therapy, and has shown to be effective against melanoma through targeting the CT antigen, NY-ESO-1. However, some of these antigens can be similar to surface proteins elsewhere in the body, causing what is known as cross-reactivity to occur. Here the T cells attack somatic cells instead as the TCR can react with the normal proteins, resulting in autoimmune toxicities. This was evident in when TCR gene therapy was used again with ACT therapy to target melanoma, but the CT MAGEA3 was used instead and fatal cardiac toxicity resulted (Hinrichs & Rosenberg, 2014).

Another challenge is that the rapidity of the T lymphocyte transfer in ACT may increase the risk of a proinflammatory immune state (Kalos & June, 2013; Yee et al., 2002). Involved in proinflammation is cytokine release syndrome (CRS), where proinflammatory cytokines are produced as a direct consequence of T lymphocyte triggering. This can lead to cytokine responses similar to that of an acute infection, hypotension, and high-grade cyclical fevers (Kalos & June, 2013; Yee et al., 2002). A second drawback to ACT is the occurrence of tumor lysis syndrome (TLS). This is a combination of metabolic complications that occur due to the destruction of large amounts of tumor. Potassium, phosphate, and nucleic acids are all released during the destruction, leading to acute renal failure. While this is not extremely prevalent, it has

been observed in the adoptive T lymphocyte transfer of chimeric antigen receptor (CAR) T cells engineered to target CD19-positive malignancies (Kalos & June, 2013).

Another major side effect is capillary leak syndrome, which results from IL-2 toxicity. The circulation of IL-2 in the bloodstream, through multiple mechanisms, causes an increase in capillary permeability and a decrease in vascular resistance (Assier et al, 2004; Pisani et al., 1991; Vial, Choquet-Kastylevsk, & Descotes, 2002). This causes the capillaries to leak some blood into the extravascular space. Eventually, this leakage leads to an accumulation of extra fluid in this space, putting the body into a hypovolemic state of decreased vascular blood volume (Vial et al., 2002; Pisani et al., 1991). Patients who suffer from this condition for extended periods of time may experience weight gain and fluid buildup in the lungs (Assier et al., 2004; Vial et al, 2002). In addition to capillary leak syndrome, IL-2 toxicity causes additional adverse side effects, such as fever, chills, and malaise (Vial et al., 2002). In addition, some practical drawbacks include: the adverse effects resulting from rigorous treatment the patient must undergo; the lab expertise required; and the labor-intensive nature of the lab work (Restifo et al., 2012; Rosenberg et al., 2008). These add more complexity and variability to the treatment plans of the clinical trials.

Finally, a major limitation of ACT that our research seeks to address is the immunosuppressive environment created by the tumor. Many of the successful research studies of ACT conclude by addressing this impediment to the efficacy of the ACT therapy (Harlin et al., 2009; Ménétrier-Caux et al., 2009; Westwood & Kershaw, 2010).

Acidic Cancer Microenvironment and Immune Cells

Physiological pH, around 7.4, is the pH at which normal cells, including immune cells, thrive and grow; lowering the pH to a more acidic pH is toxic to cells (Lardner, 2001). Cancer cells have altered metabolism and cellular processes that induce an acidic extracellular pH. Of the various cellular mechanisms that are altered, overexpression of factors involved in cellular metabolism are marked, as cancer cells undergo glycolysis at greater rates than normal cells in the human body. As tumors proliferate and grow larger, cells interior to the tumor lose access to blood vessels and are exposed to constantly hypoxic conditions (Masoud and Li, 2015).

Hypoxic conditions increase the rate of glycolysis in tumor cells. Increased glycolysis is triggered by the overexpression of hypoxia inducible factor 1 (HIF-1), a marked feature of aggressive cancer cells (Semenza, 2002). HIF-1 can cause these cancer cells to shift from oxidative phosphorylation to glycolytic metabolism and lactate production to adapt to hypoxic conditions (Denko 2008). In most healthy cells exposed to oxygen, pyruvate, an intermediate of glycolysis, is transformed into acetyl CoA. Acetyl CoA is then oxygenated in the Kreb's cycle (Cardaci & Ciriolo 2012). In cells lacking oxygen, lactate dehydrogenase converts pyruvate to lactate, which has a pKa of 3.9 at a pH of 7.4 (Phypers and Pierce 2006). Other cells in the body, especially muscle cells, produce lactate. However, this lactate production is induced by anaerobic conditions and cleared naturally from the body (Heiden et al., 2009, Romero-Garcia et al., 2016). While hypoxia does induce increased glycolysis, cancer cells produce lactic acid in both anaerobic and aerobic conditions, as established by Warburg in the early twentieth century (Warburg et al., 1927). Certain oncogenes, or mutated genes that lead to tumorigenesis, such as protein kinase B (*AKT),* and *MYC,* a transcription factor, have been indicated as potential causes for increased cancer glycolysis in aerobic conditions. The mutation of tumor suppressor genes

such as p53 have also been suggested to contribute to the Warburg effect (Kim & Dang 2006). However, its cause has not yet been fully explained (Denko 2008, Heiden et al. 2009).

In some ways, immune cells can mimic cancer metabolism and the production of lactate. Both adaptive and innate immune cells produce lactate at various points in their existence. However, their contribution of lactate to the tumor microenvironment is minimal (Romero-Garcia et al. 2016). Additionally, normally these cells can secrete the lactate out so that it can function. However, high levels of lactic acid around tumor sites have been suggested to prevent lactic acid secretion from T cells. (Fischer et al., 2007, Kato et al., 2013, Sola-Penna, 2008). This disrupts T cell metabolism and function (Kato et al., 2013; Warburg et al., 1927; Gillies et al., 2002; De Milito et al., 2010). Specifically, lactate has been found to inhibit some of the pathways of T cell stimulation, including the release of cytokines (De Milito et al., 2010; Gillies et al., 2002; Fischer et al., 2007; Lardner, 2001; Mendler et al., 2011; Kato et al., 2013; Warburg et al., 1927). The findings of these studies are significant because they indicate T cells that arrive at the tumor are suppressed by lactate, causing their cancer-killing ability to be impeded.

There is also evidence to support that immune cells either die or become deactivated because of the acidity of the microenvironment (Lardner, 2001; Kato et al., 2013; Muller, Fischer, & Kreutz, 2000; Restifo et al., 2012). An acidic microenvironment has been found to impair non-major histocompatibility complex (MHC) restricted killer cells. Natural killer cells (NK) as well as lymphokine-activated killer cells (LAK) are two types of these non-MHC restricted killer cells. They are immune cells outside of the tumor that provide an immediate immune response. Natural killer cells often respond to tumor formation quickly by detecting the loss or alteration of class I MCH molecules on the tumor cell surface while LAK cells are stimulated by the presence of IL-2. When exposed to a reduction in extracellular pH from 7.2 to

7.0, a pH found in certain human cancers, NK cells became less cytotoxic and LAK cells were not stimulated by IL-2 (Muller et al., 2000; Lardner, 2001). Furthermore, cells subjected to a more acidic pH of 6.5 were still unable to recover their cytotoxicity after a period of 24 hours (Muller et al., 2000).

Other studies also confirm that the cytotoxicity of immune cells with IL-2 activation used against multiple tumor lines were inhibited in the acidic microenvironment of cancer (Lardner, 2001; Redgeld, 1991). In one study, both mouse and human CD8+ T cells became anergic when cultured in acidic conditions ranging from pH 6.0-6.5. Subsequent buffering at physiological pH reestablished immune cell function, indicating pH as the main cause for the reduced cytokine secretion, reduced expression of adhesion molecules, and diminished activation of crucial pathways observed (Calcinotto et al., 2012). These findings highlight several weaknesses of immunotherapy in targeting cancerous tumors and are important considerations when evaluating the field of personalized cancer treatment.

The tumor environment also contributes to the inactivation of other immune-related cells, such as Myeloid Suppressor Cells (MSCs), which regulate the production of immune cells (Serafini et al., 2006). MSC cells are often active in autoimmune disease, but tumor derived factors contribute to the recruitment and maturation of these cells in order to prevent the production of CD4+ and CD8+ T cells (Serafini et al., 2006). The inhibition of LAK, NK, and other immune cells by acidity in the tumor microenvironment suggest that an acidic pH could prevent killing within the tumor microenvironment.

In addition to hindering the cytotoxicity of the aforementioned immune cells, the tumor microenvironment has also been shown to impair the movement of immune cells (Lardner, 2001). In polymorphonuclear leukocytes, or neutrophils, a type of white blood cell, locomotion

and chemotaxis were impaired at acidic pH (Lardner, 2001). In additional studies, these neutrophils were shown to decrease oxygen consumption and O_2^- production by 80-90% in acidic pH (Gabig, 1979; Lardner, 2001). Overall, an acidic microenvironment can seriously impair the functions of killer cells, including those involved in ACT, in multiple ways.

While the acidic microenvironment directly impacts immune cell function, the acidic microenvironment also contributes to the advancement of the cancer to a more aggressive phenotype (Gillies et al., 2002; Rescigno et al., 2007; Robey et al., 2009; Rosenberg et al., 2008; Rosenberg, 2001). The low pH alters gene transcription and causes genomic instability within the cancer tumors (Gillies et al., 2002). As a result of this acidic environment, genes that code for pro-metastatic, pro-invasive, and pro-survival pathways are expressed (Parks, Chiche, & Pouysségur, 2011). Many studies have correlated low pH with increased cancer metastases and invasion, suggesting that the gene expression of these pathways has changed (Estrella et al., 2013; Gillies et al., 2002; Swietach et al., 2014). Increased metastasis and invasive behavior can affect the ability of CD8+ T cells to recognize and infiltrate tumors. For example, colorectal tumors with increased metastasis have been shown to have less tumor infiltrating CD8+ T cells than those without signs of early aggressive behavior (Pagès et al. 2005).

The acidic microenvironment increases cancer metastases and invasion through other means as well. The low pH induces apoptosis in the cells surrounding the tumors, which clears out a space in which the cancer can grow into and further invade the tissue. Additionally, an acidic environment increases the release of angiogenic factors into the area around the tumor, which serves as a precursor to metastasis (Robey et al., 2009; Warburg et al., 1927). Finally, the acidic environment has been shown to cause resistance to some chemotherapeutic drugs. This is because some of the chemotherapeutic drugs used to treat cancer are weakly alkaline, which

makes them less effective at a low pH and reduces their ability to kill cancer cells (De Milito et al., 2010; Gillies et al., 2002; Greco, Marples, Joiner, & Scott, 2003; Kato et al., 2013; Swietach et al., 2014; Tannock & Rotin, 1989).

All of the aforementioned inhibitions caused by an acidic tumor microenvironment prevent the immune cells from activating and penetrating the tumor, allowing the tumor to continue to grow and metastasize (Yu, 2007; Serafini et al., 2006). If this acidic microenvironment were to be manipulated to a more physiological pH, it may increase the success of immune cells in eradicating the cancer cells.

Manipulation of pH in Cancer Research

Since the acidic tumor microenvironment is a significant factor of immunosuppression, one area of research has focused on the effects of neutralizing the tumor microenvironment on metastasis. One recent finding on cancer metastasis found that the most acidic area surrounding the tumor signifies the direction in which the cancer is growing (Estrella et al., 2013). Thus, the manipulation of pH has the potential to influence cancer behavior. There are several different approaches to manipulating the pH of the tumor microenvironment. The most direct way of altering the extracellular pH is through bicarbonate delivery. The use of sodium bicarbonate ($NaHCO_3$), commonly known as baking soda, as a systemic buffer can help prevent the growth of tumors by regulating pH (Parks, Chiche, & Pouysségur, 2013).

One study used $NaHCO_3$ buffer to treat mouse models with metastatic breast cancer, resulting in an increased pH within the tumor and a reduction of metastases (Robey et al., 2009). This study was extended to mouse models that spontaneously developed prostate tumors. The results indicated the success of buffering treatments was dependent on the stage of the tumor;

systemic buffering is more successful at treating tumors at earlier stages (Ibrahim-Hashim, 2011). In separate studies, injections and oral ingestion of bicarbonate neutralized extracellular pH and reduced invasive behavior in breast cancer tumors found in mice (Robey et al., 2011). However, these methods are still in need of improvement for safety. Other compounds, such as dichloroacetate, have been combined with bicarbonate, but have diminished the effects of the treatments (Robey et al., 2011).

In a study using a mathematical model to evaluate the safety and efficacy of buffer therapy to increase extracellular pH, it was found that the volume of bicarbonate needed to increase the pH would dangerously increase the blood pH (Martin et al., 2012). This model predicted that using an alternative buffer with an optimal pK_a of 7.1-7.2 will be more effective than bicarbonate, which has a pK_a of 6.1. Buffers that may be used are cholamine chloride (pK_a of 7.1) and BES (pK_a of 7.15) (Silva et al., 2009). These buffers, however, may have side effects and toxicity *in vivo* because, unlike bicarbonate, they are not naturally produced in the body.

Overall, while not completely understood, the Warburg effect plays an important role in cancer development and progression. Because of increased glycolysis, tumors are surrounded by a microenvironment with elevated levels of lactic acid and thus lower acidity. While lactic acid is involved in immunosuppression, the general acidity surrounding tumors also plays a role in inhibiting immune cell function. Manipulating pH at the sites of these tumors closer to physiological pH could therefore have important implications of the efficacy of immunotherapies.

Chapter 3: General Methods

Cell lines and Cultures

B16-OVA melanoma cells, EG-7 lymphoma cells, and OT-1 cells were provided by Dr. Zhengguo Xiao (University of Maryland, College Park, MD). OT-1 cells were isolated from OT-1 transgenic mice peripheral lymph nodes before being cultured and expanded. Cells were maintained in complete RPMI medium containing 10% FBS and minimally passaged. OVA expression was selected for by culturing with G418 (200ug/ml). Cells maintained in acidic conditions were cultured with complete RPMI medium containing 10% FBS, 10mM MES buffer, and 14mM $NaHCO_3$. Neutral conditions were maintained with RPMI medium containing 10% FBS, 10mM HEPES buffer, and 14mM $NaHCO_3$. Alkaline conditions were maintained with RPMI medium containing 10% FBS, 10mM Tricine buffer, and 14mM $NaHCO_3$. pH was adjusted using 1M HCl and 1M NaOH. Appendices A and B includes more information on the buffer system. Appendix A addresses buffer toxicity; Appendix B addresses media toxicity.

pH Range and Maintenance

For the purposes of this study, the following pH values were used: 6.4, 6.9, 7.4, 7.9, and 8.4. The acidic values of 6.4 and 6.9 were chosen because research has shown that several cancers survive better in slightly acidic environments (pH 6.9) and the extent of that acidity warranted exploration. pH 7.4 was selected to identify the effects of culturing the cells in a physiological environment. Finally, the pH conditions of 7.9 and 8.4 were chosen to see how increasing the alkalinity of the environment affected cancer cells, as a more alkaline condition could have been an optimal treatment when combined with immunotherapy.

All pH measurements were performed using a Mettler Toledo pH probe. pH values were maintained within ± 0.1 the target value using appropriate volumes of 1M NaOH and/or 1M HCl, as needed.

Appendix C includes additional information about the Mettler Toledo pH probe.

Cell Imaging

Images of cells were obtained using the digital Motic Microscope Camera with accompanying Motic Images 2.0 software

Appendix C includes additional information about the Motic Microscope Camera.

Chapter 4: *In vitro* Assessment of pH Manipulation and Cancer Cell Growth

Purpose

The first aim of this project, also referred to as "Aim 1," was to observe the effects of pH manipulation on the growth of EG-7 lymphoma and B16-OVA melanoma cells in acidic, neutral, and basic conditions.

pH Manipulation of EG-7 in vitro

Methods

EG-7 lymphoma cells were cultured in media at pHs 6.4, 6.9, 7.4, 7.9, and 8.4 for 48 hours. 100,000 cells were initially plated in each well. The pH of the medium in each well was maintained by adjusting with HCl and NaOH in well every 12 hours over a 48-hour period. All adjustments were made to ± 0.1 of the original pH value using 1M NaOH and/or 1M HCl. After 48 hours of incubation, cells were counted using a hemocytometer and trypan blue staining. Representative pictures for cells at each pH group were taken every 12 hours. Data was analyzed with a standard one-way ANOVA test using the Post-Hoc Tukey parameter adjustment. The ANOVA that was used was done through the Data Analysis Toolbox in Microsoft Excel. Based on this data, a Tukey Post Hoc test was done to assess the significance in the data through directly comparing two different means in a set of data. This test was only done with data sets where the ANOVA test returned with a p-value less than 0.05.

Results

EG-7 lymphoma cells were cultured in media at pH 6.4, 6.9, 7.4, 7.9, and 8.4 for 48 hours. **Figure 1** displays representative images of the lymphoma cells from each pH group after 48 hours of incubation.

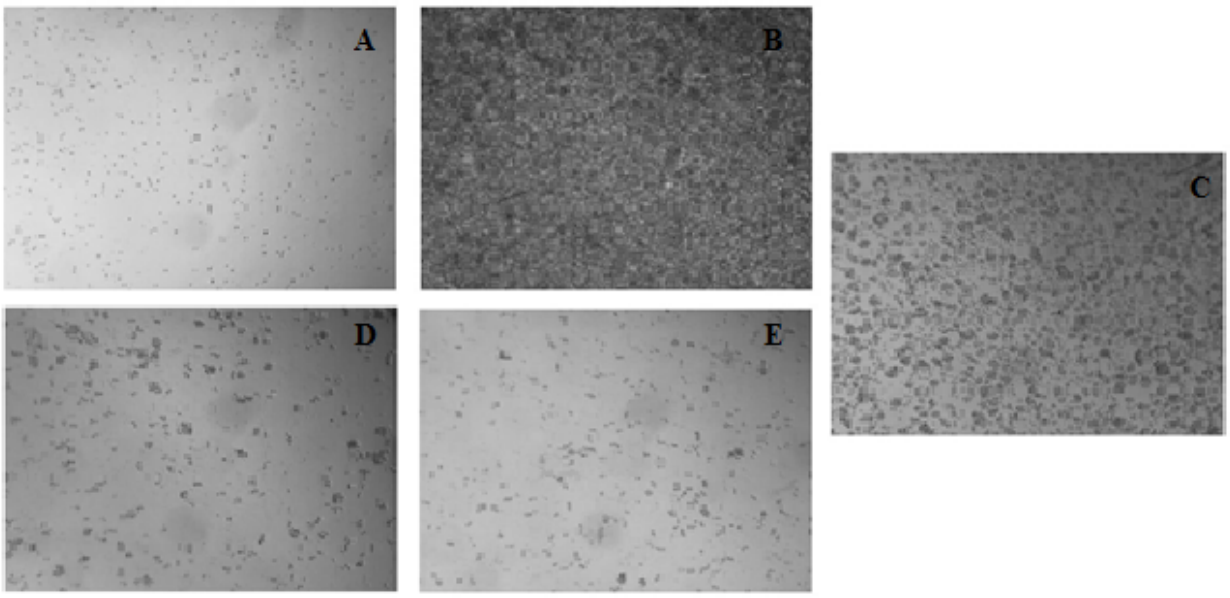


Figure 1. Representative images of the EG-7 lymphoma cells after 48 hours of incubation at pH 6.4 (A), 6.9 (B), 7.4 (C), 7.9 (D), and 8.4 (E). Pictures taken at microscope magnification 10X.

Cell counts of both alive and dead EG-7 lymphoma cells for all four wells per pH group were taken after the 48-hour incubation period. **Figure 2** provides a graphical representation of the cell count data. The error bars depicted in Figure 2 represent the calculated standard deviation for each pH group. An ANOVA test yielded an F-value of 23.439 and $p=2.63x10^{-6}$, so a Tukey Post Hoc test was performed to determine which groups were statistically different from one another. **Table 1** summarizes the significant findings from this experiment.

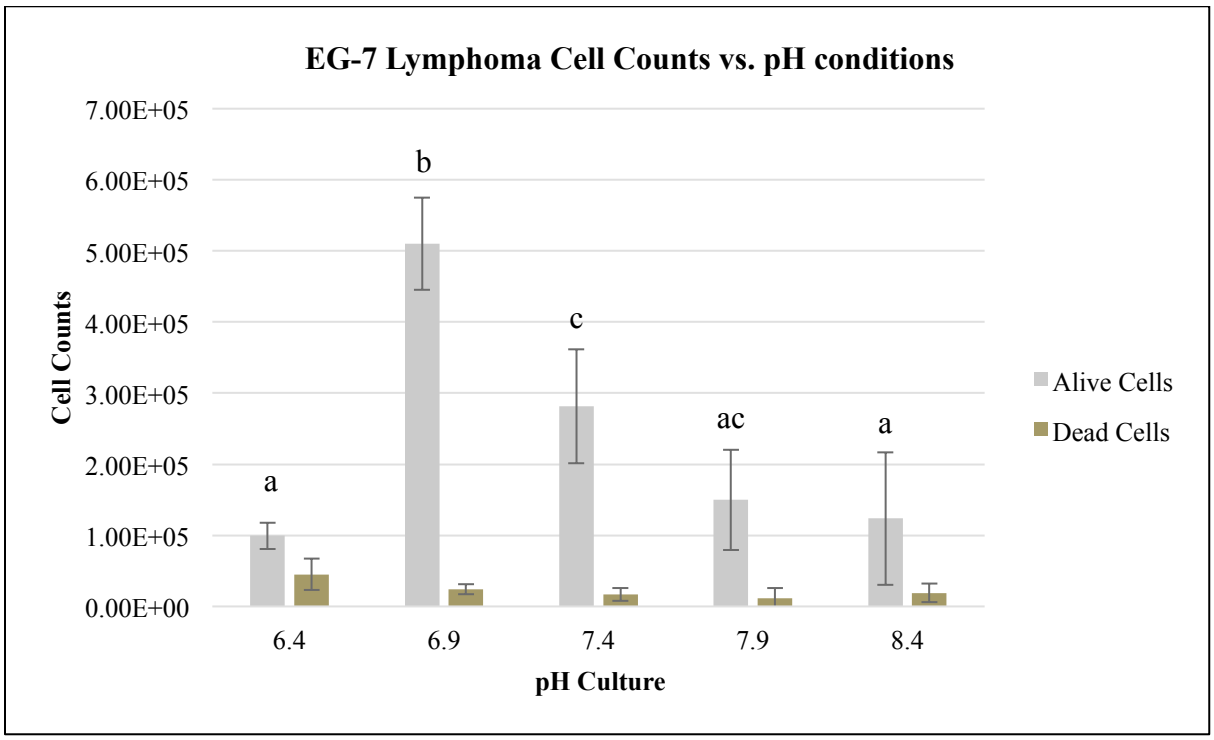


Figure 2. Cell counts of EG-7 lymphoma plated in pH media at pH 6.4, 6.9, 7.4, 7.9 and 8.4 after 48 hours of incubation. Numbers of alive and dead cells were counted using a trypan blue staining after 48 hours of incubation in respective pH media. Standard deviation was calculated and is represented by the error bars on the graph. Groups that are not statistically different from one another are designated by the same letter and groups that are statistically different from one another are designated by different letters. No statistical difference seen between dead cell counts in each pH group.

Statistically Different pH Groups
6.4<6.9
6.4<7.4
6.9>7.4
6.9>7.9
6.9>8.4
7.4>8.4

Table 1. Statistically different groups from the Tukey Post Hoc Test in EG-7 lymphoma growth. Relationship between groups indicated by greater than or less than symbol.

The lymphoma cells appeared to grow best when cultured at a slightly acidic pH of 6.9. The cells cultured at pH 6.9 showed significantly greater growth than the pH 6.4, 7.4, 7.9, and 8.4 cultures **(Table 1)(Figure 2).**

Time Interval pH Manipulation of EG-7 Lymphoma in vitro

Methods

To confirm that differences in cell survival were due to sustained pH conditions, rather than due to pH shock of cells in differing pH media, cell counts were also taken in various time periods following media pH adjustment. EG-7 lymphoma cells were cultured in media at pH 6.4, 7.5, and 8.6 for 10 minutes, 1 hour and 3 hours and then returned to regular media and allowed to incubate for 48 hours. After 48 hours of incubation, cells were counted using a hemocytometer and trypan blue staining. Student's two-tailed unpaired t-tests were used to compare every combination of experimental groups and to assess significance at $p=0.05$. Data was analyzed with a standard one-way ANOVA test using the Post-Hoc Tukey parameter adjustment. The ANOVA that was used was done through the Data Analysis Toolbox in Microsoft Excel. Based on this data, a Tukey Post Hoc test was done to assess the significance in the data through directly comparing two different means in a set of data. This test was only done with data sets where the ANOVA test returned with a p-value less than 0.05.

Results

EG-7 lymphoma cells were cultured in media at pH 6.4, 7.5, and 8.6 for 10 minutes, 1 hour and 3 hours and then returned to regular media and allowed to incubate for 48 hours. **Figure 3** displays average cell count data per pH group and incubation time collected after the cells were returned to regular media for 48 hours. Standard deviation was calculated for each pH

group and included as error bars on the bar graphs displayed in **Figure 3**. The ANOVA tests for the live cell counts yielded $F(2,9)=12.615$ and $p=0.0025$ for the 10 minute shock period and yielded $F(2,9)=10.210$, $p=0.0048$ for the 3 hour shock period, so a Tukey Post Hoc test was performed to determine which groups were statistically different from one another within these two test conditions. The ANOVA test for live cell counts after the 1 hour shock period yielded $p=0.974$, indicating no statistical difference between groups. The ANOVA test for the dead cell counts yielded $F(2,9)=7.893204$, $p=0.010474$ for the 10 minute shock period, $F(2,9)=9.176471$, $p=.006723$ for the 1 hour shock period, and $F(2,9)=1.390244$, $p=.297753$ for the 3 hour shock period. The dead cell counts at the 3 hour time point showed no statistical variance across the different pH conditions. Statistical differences between groups are represented in **Figure 3.**

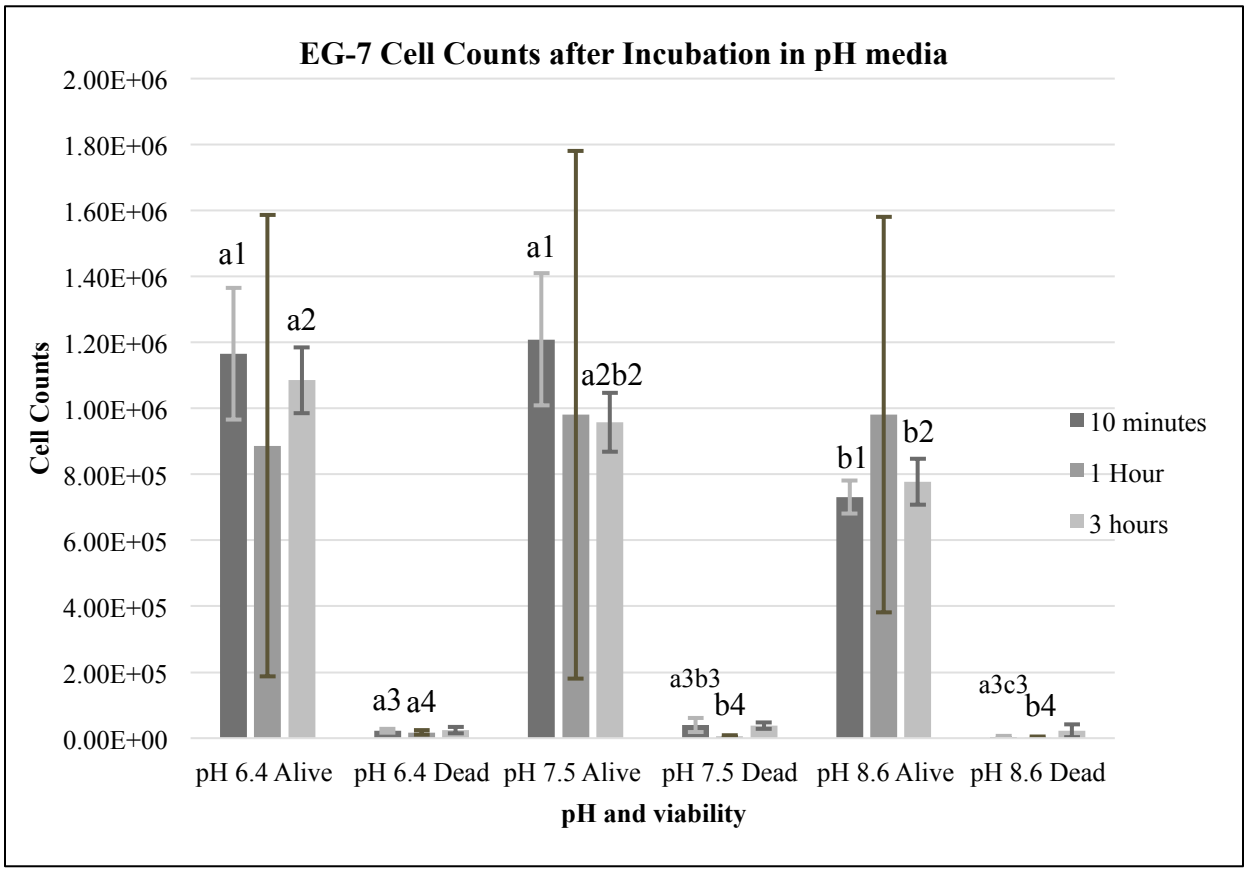


Figure 3. Cell counts taken after incubating EG-7 lymphoma cells in each pH (6.4, 7.5, and 8.6) for 10 minutes, 1 hour and 3 hours. After incubation periods, the EG-7 cells were returned to regular cell culture media for 48 hours and then counted using trypan blue staining. Error bars represent the standard deviation calculated for each pH group. Groups that are not statistically different from one another are designated by the same letter/number combinations and groups that are statistically different from one another are designated by different letter/number combinations.

A ten minute incubation period led to a statistically greater lymphoma growth in pH 7.5 and pH 6.4 cultures when compared to cells cultured at pH 8.6. There was no statistical difference in growth between cells cultured in pH 7.5 versus pH 6.4. After a one-hour incubation period, there was no statistical difference shown between any two-test groups. The results obtained after the three-hour incubation showed that there was significantly greater lymphoma growth at pH 6.4 compared to the cells cultured at pH 8.6. There was no statistical difference between cells cultured at pH 7.5 versus 6.4 and pH 7.5 versus pH 8.6.

pH Manipulation of B16-OVA Melanoma in vitro

Methods

B16-OVA melanoma cells were cultured in media at pH 6.4, 6.9, 7.4, 7.9, and 8.4 for 48 hours. 50,000 cells were initially plated in each well. The pH media in each well was maintained by adjusting with HCl and NaOH in well every 12 hours. All adjustments were made to ± 0.1 of the original pH value using 1M NaOH and/or 1M HCl. After 48 hours of incubation, cells were counted using a hemocytometer and trypan blue staining. Representative pictures for cells at each pH group were taken every 12 hours. Data was analyzed with a standard one-way ANOVA test using the Post-Hoc Tukey parameter adjustment. The ANOVA that was used was done through the Data Analysis Toolbox in Microsoft Excel. Based on this data, a Tukey Post Hoc test was done to assess the significance in the data through directly comparing two different means in a set of data. This test was only done with data sets where the ANOVA test returned with a p-value less than 0.05.

Results

B16-OVA melanoma cells were cultured in media at pH 6.4, 6.9, 7.4, 7.9, and 8.4 for 48 hours. **Figure 4** displays representative images of the melanoma cells from each pH group after 48 hours of incubation.

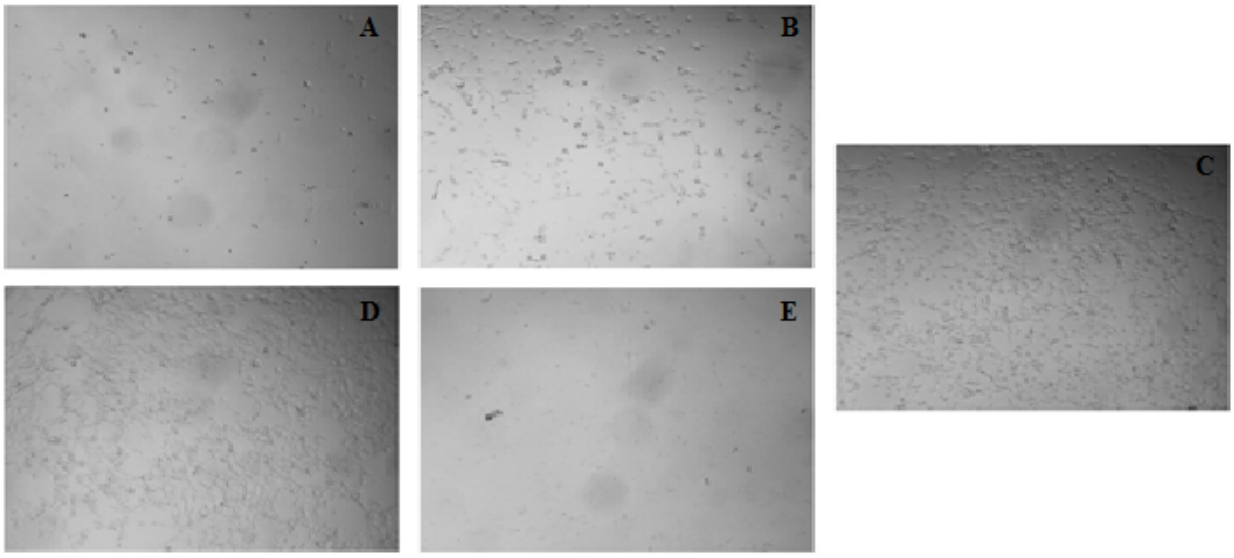


Figure 4. Representative images of the B16-OVA melanoma cells after 48 hours of incubation at pH 6.4 (A), 6.9 (B), 7.4 (C), 7.9 (D), and 8.4 (E). Pictures taken at microscope magnification 10X.

Cell counts of both alive and dead B16-OVA melanoma cells for all four wells per pH group were taken after a 48-hour incubation period. These cell counts were averaged for each pH group and the standard deviation was calculated as shown by the error bars in **Figure 5.** An ANOVA test yielded an F-value of 47.491 and $p=2.42x10^{-8}$, so a Tukey Post Hoc test was performed to determine which groups were statistically different from one another. **Table 2** summarizes the significant findings from this experiment.

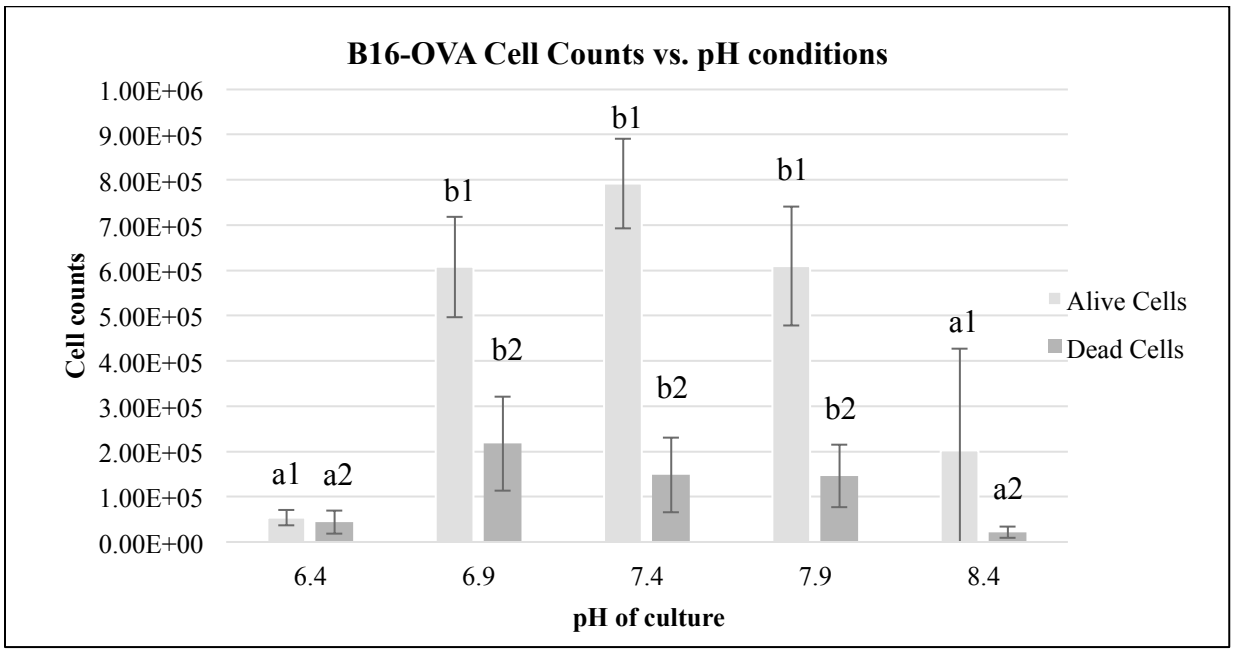


Figure 5. Cell counts of B16-OVA melanoma plated in pH media at pH 6.4, 6.9, 7.4, 7.9 and 8.4. Numbers of alive and dead cells were counted using a trypan blue staining after 48 hours of incubation in respective pH media. Standard deviation was calculated and is represented by the error bars on the graph. Groups that are not statistically different from one another are designated by the same letter/number combinations and groups that are statistically different from one another are designated by different letter/number combinations.

Statistically Different pH Groups
6.4 < 6.9
6.4 < 7.4
6.4 < 7.9
6.9 > 8.4
7.4 > 8.4
7.9 > 8.4

Table 2. Statistically different groups from the Tukey Post Hoc Test in EG-7 lymphoma growth. Relationship between groups indicated by greater than or less than symbol.

The greatest amount of melanoma cell growth appeared to be in the pH 7.4 (physiological) culture. The cells cultured at pH 6.4 and pH 8.4 showed significantly less growth

than all of the other pH cultures (6.9, 7.4, and 7.9). There was no statistical difference between the pH 6.4 and pH 8.4 cultures, the pH 6.9 and pH 7.4 cultures, the pH 6.9 and 7.9 cultures, and the pH 7.4 and 7.9 cultures **(Table 2 and Figure 5).**

Chapter 5: *In vitro* Assessment of pH Manipulation and CTL Survival, Activation, and Migration Ability

Purpose

The second aim of this project was to determine the effects of pH on immune cell activation, killing ability, and migration ability. OT-1 cells were stimulated and cultured at acidic, physiological, and basic pH conditions and analyzed using Flow Cytometry. In addition, a migration assay was performed with stimulated OT-1 cells using chemoattractant adjusted to different pH levels.

CTL Survival and Activation

Methods

Prior to isolation of OT-1 cells from OT-1 mice, 24-well plates were coated with Dimer X and B7-1 protein and allowed to incubate overnight. Following coating, plates were pulsed with SIINFEKL peptide for 2 hours. Isolated OT-I cells were then plated in media with pH of 6.4, 6.9, 7.4, 7.9, and 8.4 for 48 hours. At time of plating, a solution containing IL-2 and IL-12 cytokine signaling molecules was added to each well for stimulation. The pH media in each well was maintained as previously described every 12 hours. Representative pictures for each pH group were taken every 12 hours during the 48-hour incubation period.

After culturing, cells were harvested and stained. Surface and intracellular antibody cocktails were added to the cells and incubated for 30 min at 4°C. The stained samples were sorted and analyzed with FACSCalibur™ Flow Cytometer and CELLQUEST™ Software for percent and total OT-1 cells in the sample and expression of interferon gamma (IFN-ɣ),

Granzyme B (GzB), and IL-2 receptor chain CD25. Appendix D includes specifics about the antibody cocktails that were prepared.

Results

pH manipulation affects CTL proliferation

Cells cultured at pH 7.4 exhibit a more prominent clustering pattern and growth compared to the other cell cultures (**Figure 6c)**. Cells in the pH 6.4 and pH 8.4 cultures appear to be fewer in number and have less clustering than seen in the pH 6.9, pH 7.4, and pH 7.9 cultures. This clustering is a healthy indication of CTL activation. **Figure 6(a-e)** displays representative images of immune cells cultured for 48 hours in each pH group.

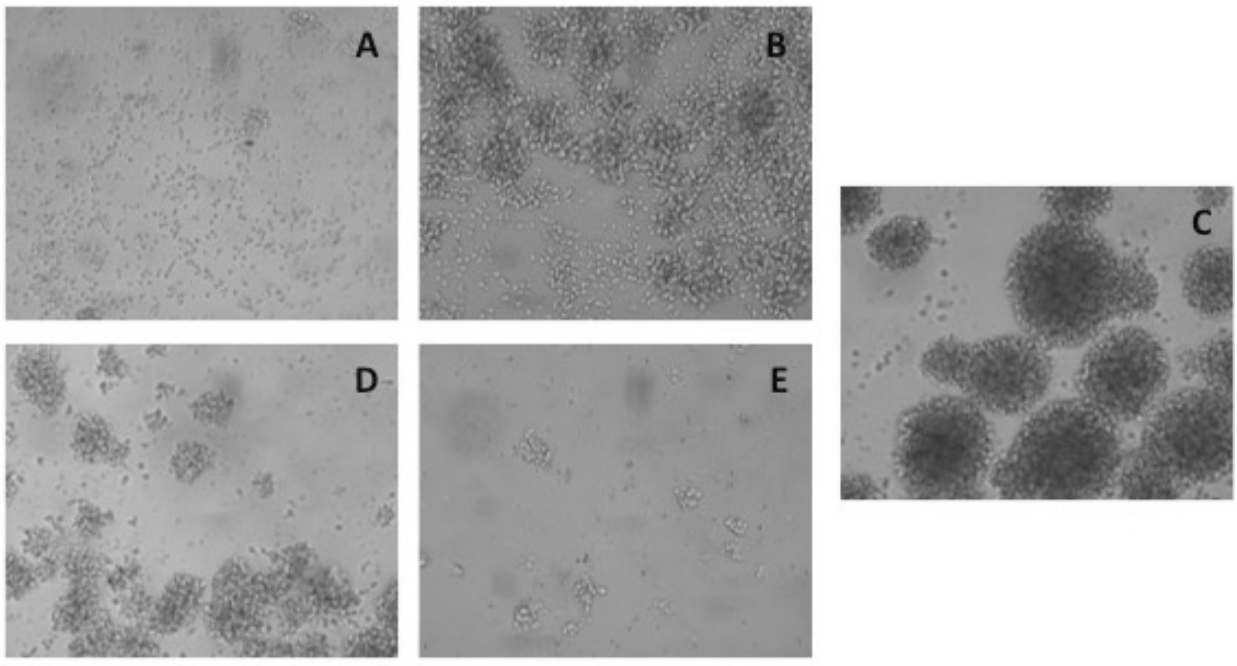


Figure 6. Representative images of OT1 CD8+ T cells after 48 hours of stimulation in pH of 6.4 (A), 6.9 (B), 7.4 (C), 7.9 (D), and 8.4 (E). Pictures taken at microscope magnification 10X.

Quantitative cell counts of alive, dead, and fold expansion were taken by the Flow Cytometer (**Figure 7a-c**). pH of 7.4 has the greatest number of alive cells and the greatest fold

expansion (**Figure 7a-b)**. More acidic of basic pH from 7.4 show decreased alive cells and fold expansion. The experimental group cultured at pH 8.4 resulted in the lowest number of alive and dead cells. These cells also had the lowest fold of expansion when compared to the other cell cultures **(Figure 7a-c).**

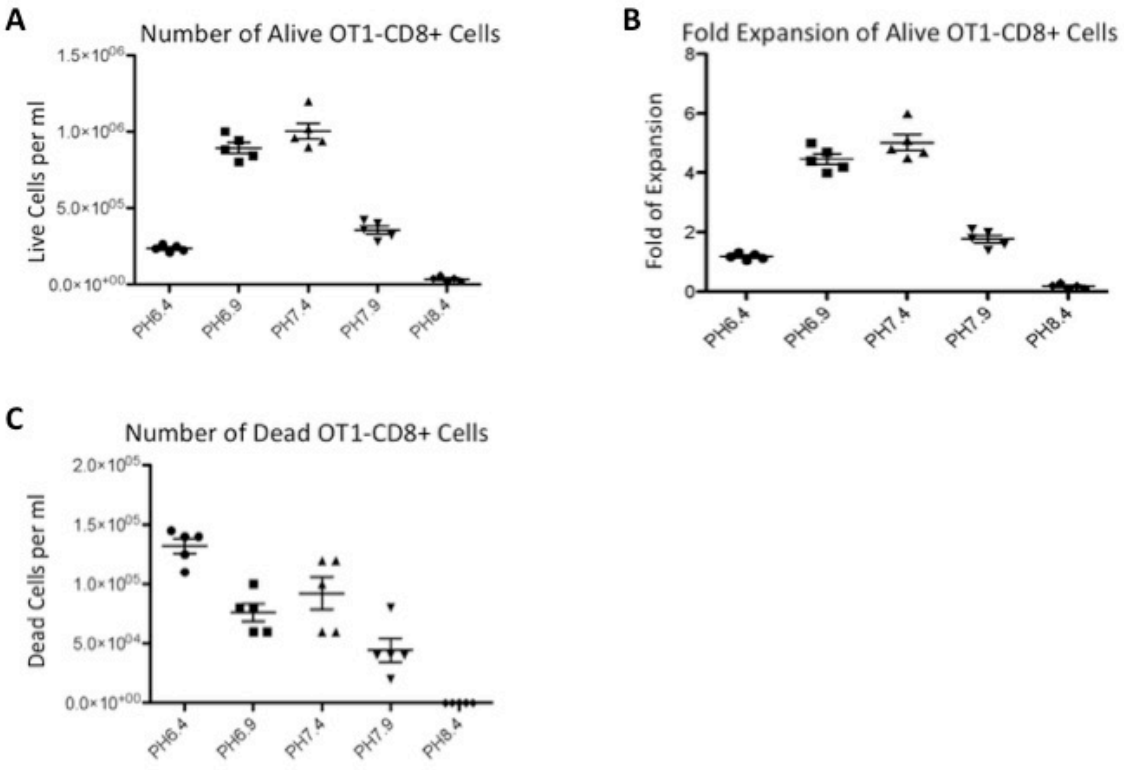


Figure 7. Relative magnitude of alive and dead OT1 CD8T+ cells, and ratio of expansion for the immune cells cultured at each pH value. Cells were initially plated at 300,000 cells per well. Graph (A) displays the number of alive cells per mL for each pH value, graph (B) displays the ratio of expansion for each set of cells, and (C) shows the number of dead cells per mL detected for each pH group.

Statistical analysis was performed on the Fold Expansion data in **Figure 7b**. An ANOVA test yielded $F(4,20)=183.947$ and $p=2.866$, and Tukey showed statistical difference between various groups. These differences are displayed in **Table 3**. These statistics support the conclusion that there is the greatest fold expansion at pH 7.4.

Statistically Different pH Groups
6.4 < 6.9
6.4 < 7.4
6.4 > 8.4
6.9 > 7.9
6.9 > 8.4
7.4 > 7.9

Table 3. Statistically different groups from Tukey Test in CTL Fold Expansion. Relationship indicated by a greater than or less than symbol.

pH manipulation affects CTL activation markers

Flow Cytometry was used to observe IFN-γ, GZB and CD25 expression by CTLs in each pH group. The presence of these three markers are positive indicators of immune cell activation and function. Representative histograms are show in **Figure 8**. The histograms display the intensity of fluorescence signal detected for the respective staining versus the number of events detected, with each cell being one event. A spike or curve indicates a large number of cells that have been detected with specific fluorescence. Respective percentages of cells for each pH group are displayed. This is an indication of protein expression and therefore activation.

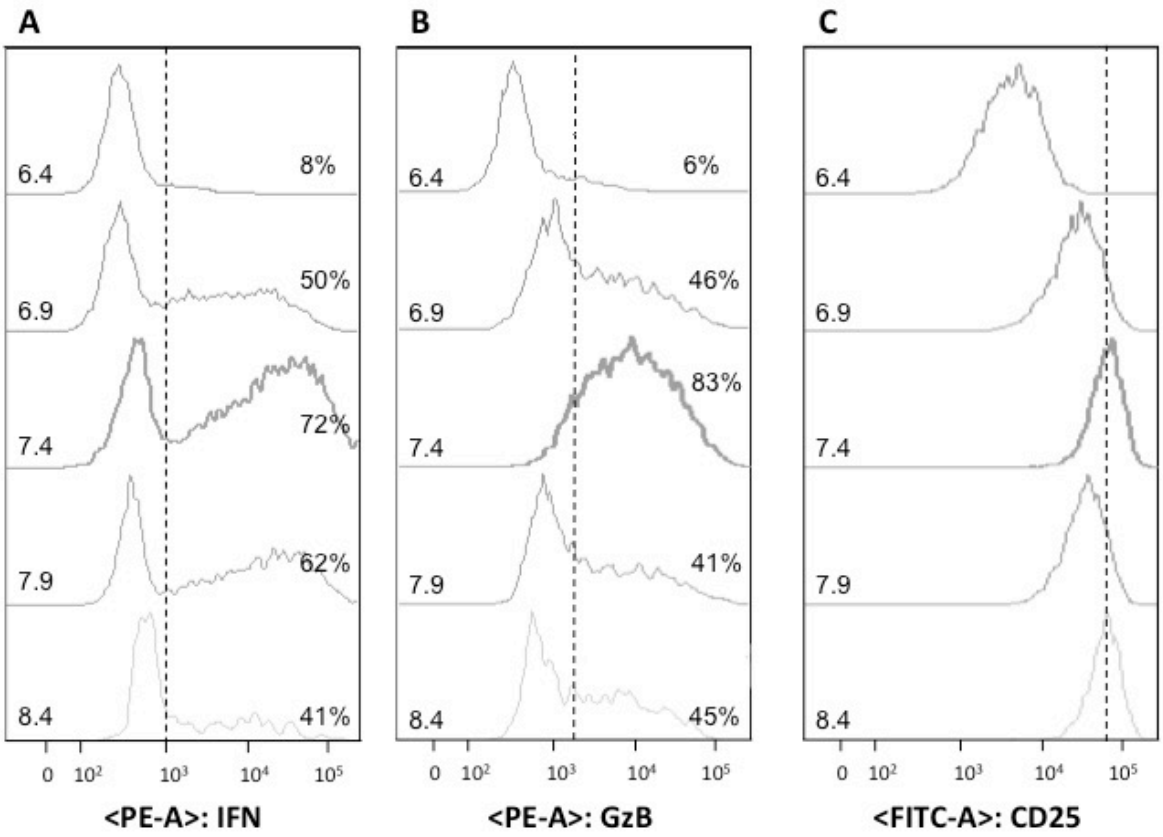


Figure 8. Comparison of expression of IFN-γ (A), GzB (B), and CD25 C) for OT1-CD8+ T cells cultured at pH 6.4, 6.9, 7.4, 7.9, and 8.4. Percentages shown for (A) and (B) only. Results are presented in a vertical chronology of pH group. X-axis is a log-scale of the intensity of fluorescence detected and Y-axis is the number of cells with that intensity of fluorescence. The left side of the dotted line on each curve is regarded as a negative intensity, while the right side of the line indicates a positive result of intensity.

Figure 8a-b displays the relative percent activation of IFN-γ and GzB in the cells at each pH. Differences in expression of these three molecules were observed in different pH conditions. At pH 7.4, IFN- γ is expressed in 72% of the cells compared to 8%, 50%, 62%, and 41% for cells cultured in pH 6.4, 6.9, 7.9, and 8.4, respectively. GzB expression also appears to have the highest level of expression in the cells cultured at pH 7.4, with 83% expressing GzB. As 7.4 is physiological pH, these results are expected.

Analysis of CD25 expression for each pH condition is represented in **Figure 8c**. CD25 expression appears to decrease when pH is lowered to 6.9 and 6.4, while expression remains similar between pH conditions 7.4, 7.9, and 8.4.

CTL Migration

Methods

A Boyden Chamber Transwell migration assay was used to observe the migration ability of CTLs induced by B16-OVA supernatant or CCL2 in acidic or neutral surroundings. Isolated OT-1 cells were seeded on 24 well plates and stimulated with antigen, B7, IL-2, and IL-12 for 3 days. B16-OVA supernatant was obtained from B16-OVA cells grown to confluency in RPMI media and CCL2 (20 ng/mL) was obtained from R&D Systems. Both chemoattractants were adjusted to pHs 6.4, 6.9, and 7.4 with 1M HCl and 1M NaOH. 24-Well Boyden Transwell Chambers (5.0 um pore) were used to test migration. 500 ul of chemoattractant was put in the bottom of the well and $1x10^5$ OT-1 cells were put on the top. One experimental trial lasted 4 hours with both CCL2 and B16-OVA. A second trial lasted 2 and 4 hours with only B16-OVA. After each time point, OT-1 cells that migrated into the lower chamber were counted using Trypan Blue staining and a Hemocytometer. Data was analyzed with a standard one-way ANOVA test using the Post-Hoc Tukey parameter adjustment. The ANOVA that was used was done through the Data Analysis Toolbox in Microsoft Excel. Based on this data, a Tukey Post Hoc test was done to assess the significance in the data through directly comparing two different means in a set of data. This test was only done with data sets where the ANOVA test returned with a p-value less than 0.05.

The more basic conditions (pH 7.9 and pH 8.4) were not tested in this and further experiments because previous experiments showed that conditions more alkaline than

physiological pH (7.4) are extremely detrimental to immune cell survival and activation and would therefore not serve as a feasible treatment method.

Results

pH manipulation affects migration of OT-1 cells

Results from the 4 hour trial with both CCL2 and B16-OVA are represented in **Figure 9**. B16-OVA supernatant shows more overall migration than the CCL2 chemoattractant. The B16-OVA chemoattractant shows decreased cell migration in acidic environments, with the cells in the pH 6.4 environment showing significantly less migration than the cells cultured in other pH environments (6.9 and 7.4). There was no statistical difference in the migration of cells in the pH 6.9 and pH 7.4 environments.

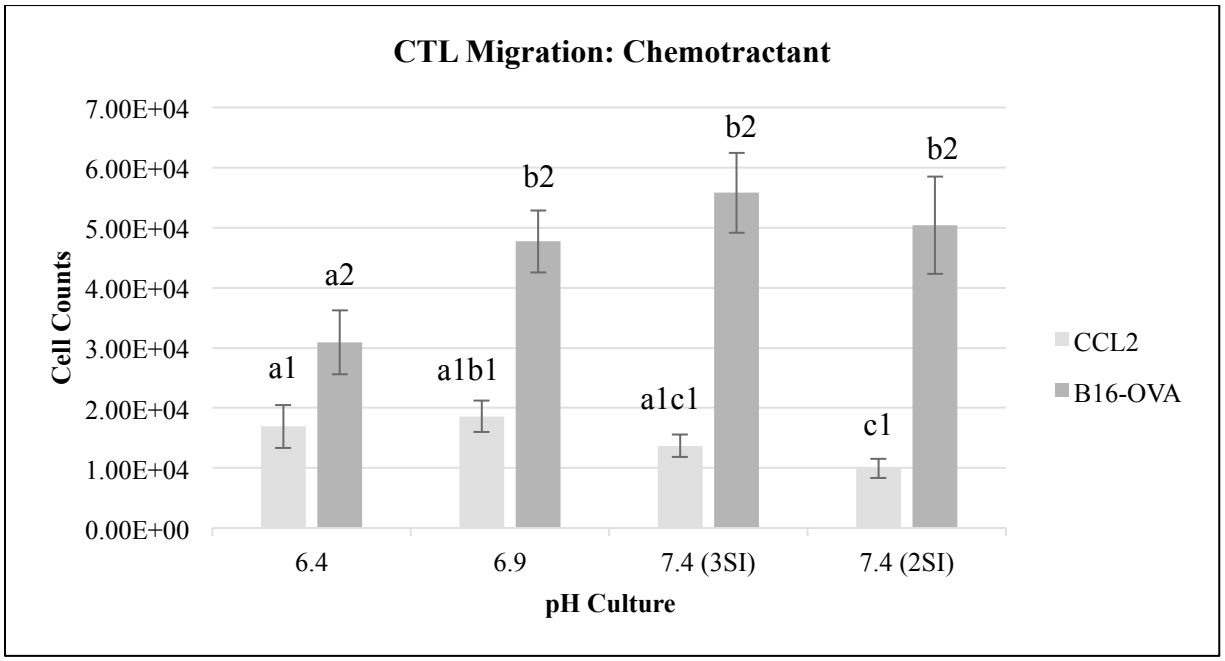


Figure 9. CTL Migration vs. pH of Chemokine. Chemokines B16-OVA and CCL2. Time point, 4 hours. Groups that are not statistically different from one another are designated by the same letter/number and groups that are statistically different from one another are designated by different letter/number combinations.

Results from the 2 and 4 hours trial with B16-OVA supernatant are represented in **Figure 10**. These results show a decrease in CTL migration when B16-OVA melanoma-associated chemokine is in an acidic environment. A significantly fewer amount of cells migrated in the pH 6.4 environment compared to the migration of the two and three-signal stimulated immune cells in the pH 7.4 environment. At four hours, the difference in the number of cells migrated in each test condition was statistically insignificant. This was further confirmed by no statistical differences found when a Tukey Test was run.

These results suggest that the migration ability of tumor cells is suppressed in an acidic environment. This could pose a drawback for immunotherapies trying to reach the tumor microenvironment.

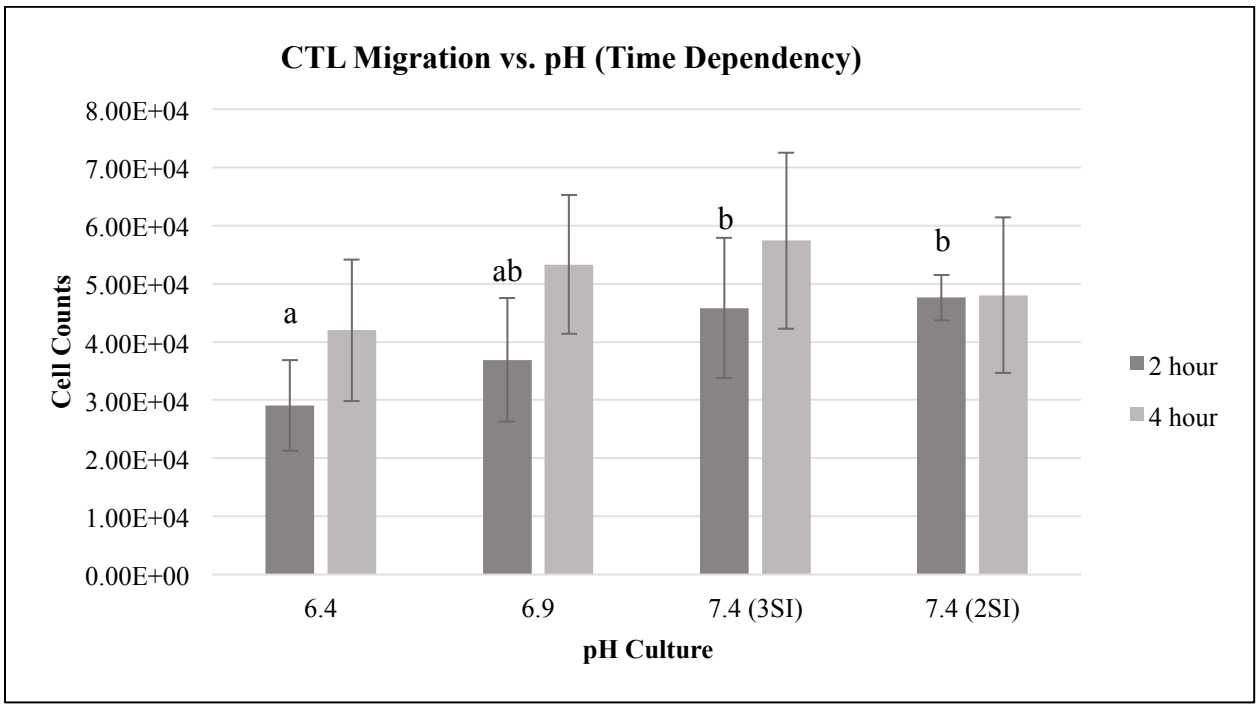


Figure 10. CTL Migration vs. pH of Chemokine using B16-OVA as the chemokine. Migration cell counts were taken at time points 2 hours and 4 hours. Groups that are not statistically different from one another are designated by the same letter and groups that are statistically different from one another are designated by different letters. No statistical difference seen between cell counts at the 4 hour time point.

Chapter 6: *In vitro* Assessment of pH Manipulation on CTL Killing Ability

Purpose

The third aim of this project was to determine the effects of pH on killing ability of CTLs against B16-OVA *in vitro.* A killing assay was performed and cell viability was determined using a CellTiter-Glo kit ® (Promega).

Aim-Specific Methods

CellTiter-Glo ® Killing Assay

CellTiter-Glo® kit quantifies cell viability through light generated in the reaction of luciferase with ATP. This reaction uses ATP to oxygenate luciferin and generate light. This light is measured in Relative Light Units (RLU), which corresponds to the number of viable cells in a culture. This is because living, viable cells produce large amounts of ATP. Therefore, the number of viable B16-OVA cells after *in vitro* treatment with CTLs can be quantified, and cell viability between experimental groups post-treatment can be compared (Zhang et al., 2014; Promega, 2015). Once RLU is determined, the percent killing can be determined by the following equation:

$$\%\ Killing = \frac{(RLU\ of\ untreated\ B16OVA) - (RLU\ of\ CD8^{+}\ treated\ B16OVA)}{RLU\ of\ untreated\ B16OVA}$$

This assay was conducted with three different experimental set-ups.

pH Manipulation During Killing and Effector – Target Ratio Optimization

Methods

B16-OVA melanoma cells were cultured in two separate groups for 10 days prior to the start of the experiment. One experimental group was cultured at a pH of 7.4, with pH maintained daily. The second experimental group was cultured in pH-unadjusted RPMI media, which was changed once daily. G418 was added daily at a concentration of 200ug/mL to both groups to select for OVA expression. At the start of the experiment, B16-OVA cells were plated in opaque-walled 96 well plates at 30,000 cells per well for each pH group (6.4, 6.9, 7.4, and unadjusted) and incubated at 37˚C, 5% CO_2 for 15 hours prior to the addition of CTLs.

To prepare the three-signal (3SI) stimulated CTLs, isolated OT-1 cells were seeded on 24 well plates and stimulated in unadjusted-pH media with SIINFEKL peptide, B7, IL-2 and IL-12 for 3 days. The control groups in this experiment were the two-signal (2SI) cell cultures, to which no IL-12 was added. *In vitro* activated CTLs were then harvested and added to the 96 well plate at various pHs, ratios to B16-OVA, and stimulation levels (2SI and 3SI) **(Tables 4 and 5)**.

Groups	CTL original pH (3 days)	B16-OVA original pH (7 days)	B16-OVA Plating pH (15 hours prior to treatment)	pH at killing	Ratio of CTLs to B16-OVA
#1 (2SI)	Unadjusted	7.4	6.4	6.4	5:1
#2 (2SI)	Unadjusted	7.4	6.9	6.9	5:1
#3 (3SI)	Unadjusted	7.4	6.4	6.4	5:1
#4 (3SI)	Unadjusted	7.4	6.9	6.9	5:1
#5 (3SI)	Unadjusted	7.4	7.4	7.4	5:1

Table 4. Experimental and control group for pH manipulation during killing

Groups	CTL original pH (3 days)	B16-OVA original pH (7 days)	B16-OVA Plating pH (15 hours prior to treatment)	pH at killing	Ratio of CTLs to B16-OVA
#6 (2SI)	Unadjusted	Unadjusted	Unadjusted	Unadjusted	5:1
#7 (3SI)	Unadjusted	Unadjusted	Unadjusted	Unadjusted	1:1
#8 (3SI)	Unadjusted	Unadjusted	Unadjusted	Unadjusted	2:1
#9 (3SI)	Unadjusted	Unadjusted	Unadjusted	Unadjusted	5:1
#10 (3SI)	Unadjusted	Unadjusted	Unadjusted	Unadjusted	10:1
#11 (B16-OVA only)	NO CTLs	Unadjusted	Unadjusted	Unadjusted	NONE

Table 5. Experimental and control groups for effector: target ratio optimization

After overnight incubation, wells were washed three times with RPMI media and visualized under a microscope to ensure CTLs were removed and B16-OVA remained adhered to the bottom. Reconstituted CellTiter-Glo ® reagent was added at a 50% concentration by volume. The plate was placed on an orbital shaker at a setting of 3 for 2 minutes to lyse the cells (Promega, 2015) and relative luminescence was read using a Wallac 1640 luminometer machine, quantified as relative light units (RLU). The percent killing was then calculated using the following equation (Adapted from Zhang et al., 2014):

Results

Conducting the killing assay previously described assessed the short-term effects of pH manipulation and effector to target ratio on CTL killing ability. For the two-signal (2SI) controls, none of the pH groups appeared to exhibit variations in killing ability. However, for the three-signal (3SI) cultures with a ratio of five effector cells to one target cell, the results showed greater killing ability of the immune cells in the pH 7.4 group, 60% killing ability, when compared to the pH 6.9 group, 56% killing ability **(Figure 11).** The pH 6.4 culture appeared to exhibit a similar level of killing ability as the pH 7.4 group. The 3SI pH 6.4, 6.9, and 7.4 groups (5:1 ratio) all showed lower killing ability than the 3SI (5:1 ratio) unadjusted pH group, which had 80% killing ability **(Figure 11).**

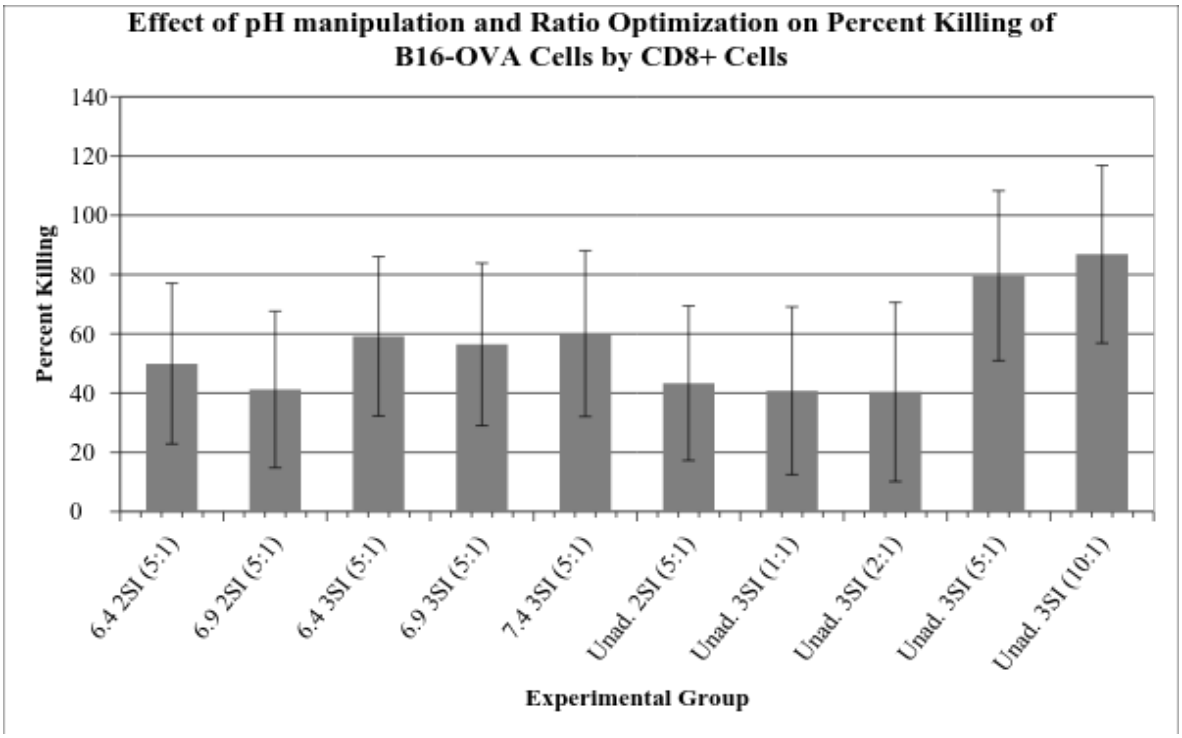


Figure 11. Comparison of percent killing. B16-OVA cells were cultured for 7 days in unadjusted media. B16-OVA cells were plated in pH media and incubated overnight. CTLs were isolated, harvested, and stimulated for three days in unadjusted media. CTLs and B16-OVA cells were plated at various ratios and killing pH levels and incubated overnight. After washing off CTLs, RLU was measured using a CellTiter-Glo ® kit. Standard deviation within each group represented by error bars.

To determine the optimal ratio of effector to target cells, a killing assay was conducted for groups with varying ratios of CTLs to B16-OVA melanoma cells. For the ratio optimization groups, little difference was seen in the killing ability of CTLs when plated in a 1:1 or 2:1 ratio. However, the 5:1 ratio of CTLs to B16-OVA melanoma cells showed greater killing ability (80%) than the 1:1 (41%) and 2:1 (40%) groups **(Figure 11)**. The 10:1 ratio of immune cells to melanoma showed a noticeably greater killing ability than the 1:1, 2:1, and 5:1 groups with 87% of B16-OVA cells killed **(Figure 11).**

Cells in a 5:1 effector to target ratio at pH 6.4 did not exhibit a difference in killing ability when grown with 2SI versus 3SI. Cells grown in a 5:1 ratio at a pH of 6.9 appeared to have a higher killing ability when grown with 3SI (56%) rather than 2SI (41%) **(Figure 11).**

pH Manipulation in Culture and at Killing

Methods

B16-OVA melanoma cells were cultured at unadjusted-pH, 6.4, 6.9, and 7.4 pH groups for 7 days prior to the start of the experiment. Media was adjusted every day and G148 was added, both as previously described. After culturing, B16-OVA cells were plated in opaque-walled 96 well plates at a concentration of 20,000 cells/well with a total volume of 100uL/well and incubated overnight.

Isolated OT-1 cells were stimulated with either three or two signals then harvested. CTLs were then plated in the B16-OVA wells in suspension in effector-to-target cell ratios of 5:1, with one control well with a ratio of 1:1. pH was maintained in the wells for killing by plating the CTLs with appropriate pH media adjusted in bottle. Wells containing only B16-OVA also served as controls **(Table 6)**. Following overnight incubation, RLU was determined as previously described.

Groups	CTL original pH (3 days)	B16-OVA original pH (7 days)	pH at killing	Ratio of activated CTLs to B16-OVA
#1 (3SI)	6.4	6.4	6.4	5:1
#2 (3SI)	6.9	6.9	6.9	5:1
#3 (3SI)	7.4	6.4	6.4	5:1
#4 (3SI)	7.4	6.9	6.9	5:1
#5 (3SI)	7.4	7.4	7.4	5:1
#6 (2SI)	Unadjusted	7.4	7.4	5:1
#7 (3SI)	Unadjusted	7.4	7.4	1:1
#8 (3SI)	Unadjusted	6.4	6.4	5:1
#9 (3SI)	Unadjusted	6.9	6.9	5:1
#10 (3SI)	Unadjusted	7.4	7.4	5:1
#11 (B16-OVA only)	NO CTLs	Unadjusted	Unadjusted	NONE
#12 (Unwashed controls, B16-OVA only)	NO CTLs	Unadjusted	Unadjusted	NONE

Table 6. Experimental groups used for pH manipulation in culture and killing.

Results

For the 3SI groups that killed at a pH of 6.4, the only difference was seen between the treatment groups for which CTLs were cultured at pH 7.4 **(6.4 Culture C, Figure 12)** and CTLs cultured in unadjusted media **(6.4 3SI 5:1, Figure 12)**. Cells cultured in unadjusted pH had a higher RLU, indicating more viable B16-OVA cells and less killing by the unadjusted CTLs than the CTLs adjusted to pH 7.4 for three days (33% versus 20% respectively) **(Figures 13, 14).**

For the 3SI groups that killed at a pH of 6.9, a difference in killing ability was found between the treatment groups in which CTLs were cultured at pH 7.4 **(6.9 Culture C, Figure 13)** and CTLs cultured in unadjusted media **(6.9 3SI 5:1, Figure 12)**. The group cultured at pH 7.4 prior to plating appeared to exhibit less killing ability than the treatment with CTLs cultured in unadjusted media **(Figure 12)**.

There was no drastic difference between the 2SI control **(7.4 2SI, Figure 12)** and the 3SI group that was cultured in unadjusted media and killed at pH 7.4 with 5:1 effector to target cell ratios **(7.4 3SI 5:1, Figure 12)**.

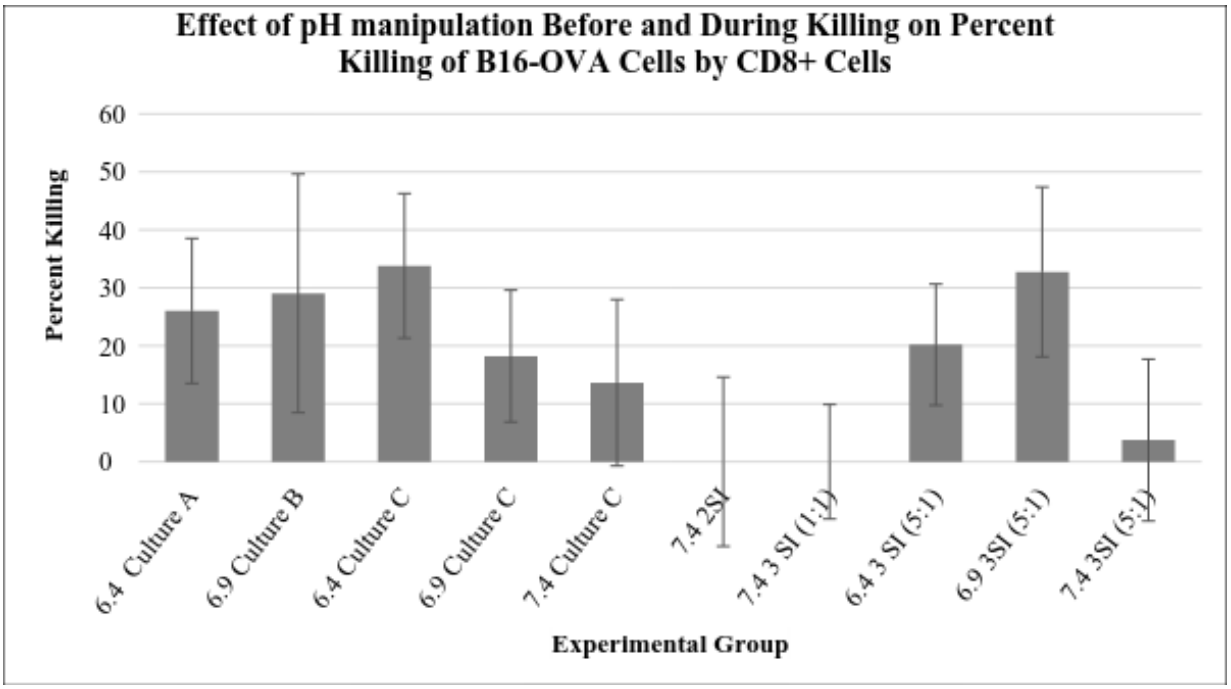


Figure 12. Comparison of percent killing. B16-OVA cells were cultured for 7 days in pH media. Media was adjusted with 1M HCl and NaOH in bottles and culture media was changed once a day. CTLs were isolated, harvested, and stimulated for three days. CTLs were cultured in pH media for 3 days and adjusted once per day with 1M HCl and NaOH. Culture A CTLs were cultured in 6.4, Culture B in 6.9, and Culture C in 7.4. All other groups were stimulated and cultured in unadjusted media. CTLs and B16-OVA cells were plated at a effector to target cell ratio of 5:1 (with the exception of a 3SI control maintained at pH 7.4) and incubated overnight. After washing off CTLs, RLU was measured using a CellTiter-Glo ® kit. Standard deviation is shown in error bars.

Naïve CD8+ T Cell Killing Assay

Methods

A third killing assay was done with naïve T cells. Naïve T cells have not been stimulated with any of the three signals and should therefore not be able to kill. B16-OVA melanoma cells were cultured for 4 days in pH media (6.4, 6.9, and 7.4) and plated at 20,000 cells/well. Naïve

OT-1 cells were plated onto the B16-OVA cells at a ratio of 5:1 and incubated overnight **(Table 7)**. RLU for the naïve cells was determined as previously described.

Groups	B16-OVA original pH (4 days)	pH at time of treatment	Ratio of naïve T cells to B16-OVA cells
#1	6.4	7.4	5:1
#2	6.9	7.4	5:1
#3	7.4	7.4	5:1
#4	Unadjusted	Unadjusted	None Added

Table 7. Experimental groups used for naïve CD8+ T cell killing assay

Results

The killing ability of naïve cells appears to be extremely low (<1%) at the pH conditions tested. The killing ability of the cells cultured at pH 6.4 appeared to be higher than that of the cells cultured at pH 6.9 and pH 7.4, with the cells cultured at pH 6.9 exhibiting the lowest amount of killing ability relative to pH conditions 6.4 and 7.4 **(Figure 13)**.

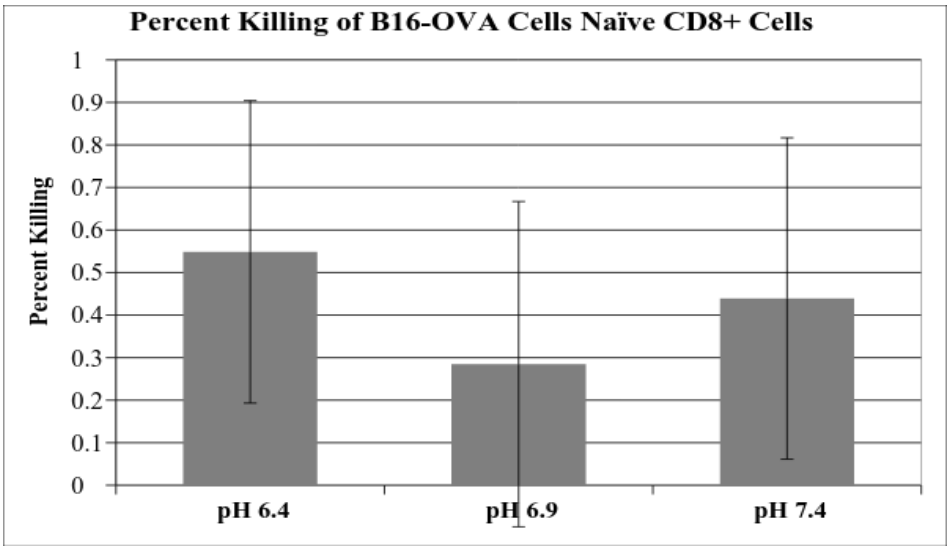


Figure 13. Naïve killing assay, comparison of percent killing. B16-OVA cells were cultured for 4 days in pH media. Media was adjusted with 1M HCl and NaOH in bottles and culture media was changed once a day. OT-1 cells were isolated but were not stimulated. Naïve OT-1 cells and B16-OVA cells were plated at an effector to target cell ratio of 5:1 and incubated overnight. After washing off naïve OT-1 cells, RLU was measured using a CellTiter-Glo ® kit. Standard deviation represented by error bars on the graph.

Chapter 7: Discussion

Aim 1: In vitro assessment of pH manipulation and cancer cell growth

The goal of aim 1 was to assess the effects of environmental pH on cancer cells *in vitro*. In previous work it was demonstrated that human melanoma cells cultured in an acidic pH of 6.8 were more invasive and had increased angiogenic potential in comparison to cells cultured at a physiological pH of 7.4 (Rofstad et al., 2006). It was also shown that treating mice with breast cancer using sodium bicarbonate reduced breast cancer metastases (Robey et al., 2009). However, not many studies demonstrated the effects of pH on growth or looked into the effects of alkaline conditions.

We found that proliferation of melanoma and lymphoma was considerably inhibited by alkaline growth conditions of pH 8.4. This is a new finding, as most current research has not tested such extreme alkaline conditions. This result shows potential for using targeted pH manipulation to increase the pH of the cancerous microenvironment as a method to slow tumor proliferation and make tumor cells more susceptible to attack by immune cells. One avenue to explore would be a pH-neutralizing agent delivered to the site of the tumor using pH-sensitive hydrogels or nanoparticles. However, raising the tumor microenvironment to a basic pH could have detrimental side effects on the survival and homeostasis of the surrounding, non-cancerous tissue. This side effect would need to be minimized for such a delivery system to work.

In addition, results from this study demonstrated that lymphoma cells exhibited greatest growth in slightly acidic conditions (pH 6.9) compared to physiological (7.4), alkaline (7.9 and 8.4), and extreme acidic (6.4) pH media. This optimal growth at acidic conditions corroborates the pro-survival characteristics of cancer in acidic conditions, such as the ability of melanoma cells growing in low pH to establish a higher ratio of oxygen utilization to lactic acid production

than those growing close to physiological pH (Burd et al., 1990). Additionally, lymphoma cell growth was most inhibited by alkaline growth conditions, in pH 8.4 growth media. This is a new finding as most current work does not include alkaline testing conditions. Therefore, growth in physiological conditions may hamper lymphoma cancer cell growth. A recent study found that neutralizing the tumor microenvironment with bicarbonate monotherapy impaired the growth of some mouse tumors (Pilon-Thomas et al., 2015). Their findings corroborate our lymphoma growth results, suggesting that pH neutralization may serve as a strategy in a lymphoma immunotherapy approach.

To confirm that differences in cell growth were due to sustained pH conditions, rather than due to pH shock of the cells in differing pH media, cell growth was quantified after subjecting the cells to different pH conditions for 10 minutes, 1 hour, and 3 hours. These experiments exhibited diminished survival due to alkaline pH conditions after pH adjustment for 10 minutes, but no difference between acidic and neutral conditions. Survival at 1 hour was not different between any pH group, and pH 6.4 showed more survival than pH 8.6 at 3 hours. It is unlikely, however, that the amount of live cells in the pH 8.6 group would decrease as dramatically as it appears to have decreased without an equal increase in the amount of dead cells present. This discrepancy, combined with the extremely large error bars in the one-hour time point, indicates experimental error. A repeat is necessary to confirm these results.

The findings from the melanoma growth studies did not exhibit the same effects that pH manipulation appeared to have on lymphoma cells. Past studies have shown that metastasis of melanoma increases as a result of hypoxic (acidic) condition, as discussed earlier pH (Burd et al., 1990. The results from this study suggest that extreme acidic and alkaline conditions inhibit melanoma growth, while slightly acidic, physiological, and slightly alkaline conditions all allow

for cell survival and proliferation. The number of live cells counted and visualized appeared to be greatest in the culture grown at pH 6.9, 7.4, and 7.9 when compared to the more acidic (pH 6.4) and more basic (8.4) conditions. The data suggests a trend, however, of greater growth in pH 7.4 than any other pH condition. This experiment needs to be repeated to be able to distinguish optimal growth conditions between pH 6.9, pH 7.4, and pH 7.9. Past studies have shown that metastasis increases as a result of hypoxic (acidic) conditions, but have not shown differences in growth rate (Pilon-Thomas et al., 2015). Therefore, lack of difference in cell growth in different conditions may be indicative of invasive growth and decreased sensitivity to extracellular pH in the B16-OVA cell line. Also, greater aggression observed in past studies may be the only feature of hypoxic conditions in melanoma cells while proliferation is not altered. These findings can be applied to human melanoma models as the metabolism and effects of extracellular pH are similar between human melanomas and B16-OVA cells (Bellone et al, 2013).

Evidence of diminished lymphoma cell proliferation at physiological (pH 7.4) and more alkaline conditions, as well as evidence of diminished metastasis of melanoma in more acidic and more alkaline conditions in past studies, may prove to be an effective strategy for controlling tumor growth. Limiting metastasis and cell proliferation by creating a more basic condition around the tumor, however, is not efficient as a stand-alone treatment option. This treatment could potentially optimize adoptive cell transfer therapy or forms of chemotherapy and increase their efficacy in patients. These results also suggest that the effects of a combined treatment of pH manipulation and ACT would more likely stem from the effect of pH on the immune system's ability to function and kill cancer rather than the pH manipulation itself killing the cancer cells directly.

Aim 2: In vitro assessment of pH manipulation effects on immune cell survival, activation and migration

It appears that $CD8^+$ T cell survival, activation, and migration is best in physiological conditions (pH 7.4) when compared to more acidic and more basic environmental conditions. It has been shown in past studies that the acidic microenvironment of tumors may itself be a mechanism of immune escape (Bellone, 2013). This study assessed the effects of pH manipulation on activated immune cell survival, and showed that cell growth and fold expansion is greatest in physiological conditions. Qualitatively, it appears that the cells grown in physiological conditions exhibited a greater cell density and clustering pattern than cells grown in more acidic and basic conditions. Cells cultured in more acidic and more basic conditions showed decreased cell numbers compared to the cells grown at physiological pH of 7.4, suggesting that both alkaline and acidic environmental conditions, relative to 7.4, are detrimental to T cell growth. Fold expansion, on the other hand, was shown not to be significantly different between pH 6.9 and 7.4, but these groups were significantly different from the more acidic (pH 6.4) and basic (pH 8.4) groups. This indicates that T cell proliferation is hindered at either extreme basic or extreme acidic conditions.

Flow cytometric analysis was conducted to assess the effect of pH manipulation on the activation of the $CD8^+$ T cells cultured in pH 6.4, 6.9, 7.4, 7.9, and 8.4. A comparison of cytotoxic effector and receptor expression between the different cell cultures shows that the level of activation of the $CD8^+$ T cells does differ with respect to the pH of the cellular microenvironment. IFN- γ is cytokine produced by activated immune cells to strengthen the immune system response and Granzyme B (GZB) is a cytokine responsible for inducing apoptosis in target cells. Expression of these molecules by activated $CD8^+$ T cells serves as an

indication of the T cell's killing ability. Of the pH conditions tested, the percent expressions of IFN- γ and GZB in the population of cells cultured at pH 7.4 are the highest. This suggests that a greater proportion of T cells are better suited to kill target cells when they are activated at a physiological pH in comparison to the cells activated in more acidic and more basic conditions.

The data obtained from this study also indicates that T cells cultured in very acidic conditions (pH 6.4) and very basic conditions (pH 8.4) do not possess substantial killing ability. The percentage of total cells expressing both IFN-γ and GZB cytokines in T cells activated at pH 6.4 and pH 8.4 are very low compared to the cells activated in less acidic conditions. These two extreme conditions also showed very little cell survival. These data suggest that acidic conditions are detrimental to $CD8^+$ T cell killing ability and survival. Similar results have also been found in other papers. A recent study mentioned previously also found that acidic conditions decreased IFN-γ production (Pilon-Thomas, 2015). Another group demonstrated that lactic acidosis inhibits cytokine production (Mendler et al., 2011). In addition, an extremely alkaline microenvironment appears to be detrimental to $CD8^+$ T cell survival, activation, and killing ability. Alkaline environments such as the ones tested in this project are not common in literature.

After determining how pH manipulation impacts immune cell activation, the effect on the migration ability of OT-1 cells towards melanoma-associated chemokines was tested. It was found that cell migration was decreased when the chemoattractants were present in an acidic environment. These results suggest that the migration ability of T cells is suppressed in a more acidic environment and that a physiological environment seems to be the optimal environment for immune cell migration. These results would suggest that the acidic microenvironment of a tumor inhibits the migration of immune cells toward the tumor. This poses a potential drawback for immunotherapies such as ACT because the reintroduced immune cells could have difficulty

migrating through the tumor microenvironment and reaching the cancerous cells. However, this also suggests that neutralization of the acidic tumor microenvironment could enhance the migration of an activated T cell around and to the tumor. This could be a potential way to increase the efficacy of ACT treatment.

Previous work is consistent with these findings. Extreme acidic conditions (pH 6.0-6.5) have been demonstrated to reduce cytokine secretion as well as negatively impacting the STAT5 and ERK pathways (Calcinotto et al., 2012). In another study, CTLs were activated *in vitro* to a pH of 6.6 or 7.4 and injected into mice that had received oral bicarbonate treatment three days prior to melanoma tumor injection (Pilon-Thomas et al., 2016). The purpose of this project was to combine bicarbonate treatment with ACT in a mouse model. The results showed that the production of IFN-γ was blocked when CD8+ T cells were activated in an acidic pH, providing some explanation for this phenomenon. The authors also found that mice treated with combined therapies of oral sodium bicarbonate and immunotherapy had significantly more T cells infiltrate the tumor and long-term survival rate of the mice was 30% more than mice treated with immunotherapy alone (Pilon-Thomas et al., 2016). The combination treatment was found to cure some of the mice completely of the cancer tumor. These significant findings are very promising for the advancement in this area of cancer and immunotherapy research, and will hopefully move into clinical trials in the future.

In addition, pH manipulation has also been found to affect more than just T cells of the immune system, including the cytotoxicity of natural killer (NK) and lymphokine-activated killer (LAK) cells toward lymphoma. Acidic environments have been shown to decrease surface ligand expression in these cells as well (Fischer et al., 2000). Therefore, manipulation of pH could improve ACT by improving cytotoxic cell function as well as improve the body's general

response to tumors. The specific mechanism of immunosuppression of this environment is still unclear; though it has been shown in past studies that lactic acid could be the major immunosuppressive agent (Fischer et al., 2007; Choi, Collins, Gout, and Wang, 2013). Our study suggests that lactic acid may not in fact be the major contributor, as lactic acid levels were constant between different pH buffers. Further experiments need to be conducted to find a clear mechanism of pH-dependent immunosuppression as independent of lactic acid differences.

Aim 3: In vitro assessment of the effects of pH on CTL killing ability

The results obtained from the studies that tested the killing ability of immune cells under various conditions contained multiple inconsistencies, making the findings inconclusive. For this portion of the experiment, two killing assays were performed. In the second killing assay, the immune cells were stimulated by the three-signal pathway, cultured in unadjusted media, and placed in a physiological (pH 7.4) killing environment with a 5:1 effector to target ratio. Based on the high intensity of relative luminescence of the culture, the immune cells did not appear to kill any of the melanoma cells. This finding is inexplicable and inconsistent with data obtained from the first killing assay. For the cell cultures stimulated by the three-signal pathway with a ratio of 5 effector cells to 1 target cell, the cells cultured at pH 7.4 exhibited a higher killing ability when compared to the cells cultured at pH 6.9. The pH 6.4 group did not appear to differ in killing ability when compared to the pH 6.9 and pH 7.4 groups. It is surprising that a more extreme pH value of 6.4 would not show a significant difference in killing ability from the pH 7.4 group because the pH 6.9 group did seem to differ from the pH 7.4 group. However, the difference in percent killing was relatively small, and therefore most likely short-term pH manipulation has little effect on killing ability. Repeated trials would be necessary to confirm

this suggestion. These findings suggest that the adjusted media itself might be decreasing killing ability or that human error was involved.

As a result, the assay itself needs improvement. One such improvement could be the step in which the immune cells are washed off of the melanoma cells. The inconsistent results as well as the observations of unwashed controls that had a much higher RLU than any group (including the washed control with only B16-OVA) suggest that the wash step of the assay must be improved. It is possible that the intensity of the wash varied among the wells and may have caused the removal of B16-OVA cells and CTLs from some wells causing skewed data.

For the ratio optimization groups, the findings suggest than an effector to target ratio of 5:1 is adequate. In addition, three signals should lead to a greater amount of killing than only two signal activation. Therefore, pH 6.9 group's data was consistent with expectations whereas the pH 6.4 group was not. This could be due to an error in adjustment. However, little difference was seen between the two-signal control and the three-signal group that was cultured in unadjusted media and placed in a killing environment at a pH of 7.4 with 5:1 effector to target cell ratios. This finding, along with the other inconsistencies in the data is inexplicable and calls into question the validity and accuracy of the data. A repeat would be necessary to make conclusions from this assay. With so many experimental groups being performed simultaneously, it is also possible that test tubes were accidentally switched or mislabeled. Protocol improvements and repetition are recommended to better understand the data.

Overview of findings

The table below summarizes the findings of the experiments conducted to test the effects of pH manipulation on tumor proliferation and the cytotoxic T-lymphocyte response. Optimal

conditions are represented by a ✓ while × indicates harmful conditions for the aspect of cell survival that was studied.

	pH 6.4	pH 6.9	pH 7.4	pH 7.9	pH 8.4
Lymphoma Growth	×	✓	×	×	×
Melanoma Growth	×	×	✓	×	×
Immune Cell Survival	×	×	✓		
Immune Cell Activation	×	×	✓		
Immune Cell Migration	×	×	✓		

Table 8. Summary of findings

Limitations

This project focused on *in vitro* manipulation of pH through buffer systems. To be able to validate these findings in physiological conditions, *in vivo* data is necessary. Growth rates, migrations ability, and killing ability of melanoma/lymphoma and CD8+ T-lymphocytes are limited to largely two-dimensional systems that do not imitate the three-dimensional structure of a tumor. Furthermore, physiological conditions, such efficacy of carbonic anhydrase surrounding the tumor or decreased persistence of a basic buffer due to angiogenesis, cannot be accounted for in an *in vitro* setting.

pH measurements were taken using a Mettler Toledo pH probe. This pH probe has an error range of ±0.25. There were also a variety of experimental errors introduced in our handling of the pH probe. For example, the pH probe often fluctuated and required repeated recalibrations to obtain stable readings. For this reason, a considerable amount of time was required to adjust the cell culture medium, potentially reducing the integrity of the medium. Furthermore, adjustment of the medium in culture also required considerable time and, in some experiments, led to extended periods in which cultures were not in proper growth conditions. To mitigate this

logistic flaw, experiments were conducted with separated experimental groups in different culture plates.

Data obtained in all experiments consisted of at least three experimental repeats. However, these repeats between separate experiments were exposed to different time lapses outside of growth conditions of the incubator. Therefore, variation between experiments was not quantified and may be a source of error as well.

Lastly, when counting a small number of cells, the cells suspension was centrifuged and suspended in a decreased volume of medium. This allowed for more precise cell counts, but exposed cells to additional stress during centrifugation. This could have affected the number of live cells counted and thus, small cell counts may have larger range of error than large cell counts.

One possible way to better quantify cell proliferation is through conducting a carboxyfluorescein succinimidyl ester (CFSE) staining. For a CFSE staining, a fluorescent dye is added to an aliquot of cells and is integrated into the cell membrane. When cell proliferation occurs, traces of dye are present in replicated cells and the intensity of fluorescence decreases with subsequent replication cycles. This procedure is commonly done with immune cells and can serve as a more accurate study of how manipulating pH affects immune cell proliferation versus survival.

Chapter 8: Conclusion

The results from this study, though preliminary, could have far reaching implications. From the first part of the study, differences were observed in the growth of melanoma and lymphoma cultured at different pH levels. Lymphoma grew optimally in a slightly acidic condition, which suggests that manipulation to a physiological condition could slow its proliferation and improve patient outcomes. Melanoma responded to the various pH conditions differently than lymphoma, growing well in slightly acidic, slightly basic, and physiological pHs. These different responses to pH conditions suggest that pH-based treatments might have different effects depending on the type of cancer treated and warrants additional research.

The second part of this study demonstrated that pH manipulation could potentially impact multiple aspects of the immune response. The level of CTL activation and proliferation were greatest in physiological conditions. This indicates that CTL function could be increased if the area around a tumor could be manipulated to be less acidic, potentially allowing the body to better fight cancer cells. Furthermore, the results obtained from the migration assay suggest that acidic microenvironments suppress CTL migration. Therefore, pH neutralization of those environments could improve both the function and ability of CTLs to move toward cancerous targets.

Together, these two parts of the study indicate that neutralizing the acidic microenvironment of cancerous cells could potentially decrease cancer proliferation while allowing CTLs to better migrate to the cancerous target and to function optimally. The findings from this study lay the groundwork for a treatment combining pH manipulation and immunotherapy – an option that could have the potential to improve the immune response to cancer and subsequently improve patient outcomes.

Future Directions

To validate the findings in this project, in vivo experiments need to be conducted in mice. ACT in tumor-carrying mice must then be further assessed for efficacy across different administered buffers, as tested by Pilon-Thomas in 2015. pH manipulation has been done largely through direct administration of bicarbonate (Robey et al., 2009) or a buffer system, as this project attempted. Tail vein injections can be used to administer non-toxic basic buffers, such as bicarbonate. Metronomic, or a continuous low-dose approach, delivery of bicarbonate can also be administered throughout the course of the ACT treatment to maintain the effects of bicarbonate and avoid side effects (Coleman et al., 2008).

Administration in human subjects would, however, require a highly localized modulation of pH at the site of the tumor. Hydrogels may be an efficient way to localize the delivery of buffer or bicarbonate. Hydrogels are polymers cross-linked to one another, and can be activated by a myriad of physiological conditions, such as temperature. pH sensitive hydrogels are responsive to pH changes in the aqueous surrounding environment, and de-swell to release internal aqueous substances. pH sensitive hydrogels can be used to target the acidic microenvironment of a tumor and release chemotherapeutics along with basic buffers.

Another method to target a tumor site with pH-neutralizing buffers may be to use nanoparticles. Much like pH-sensitive hydrogels, pH-sensitive nanoparticles are responsive to higher concentrations of hydrogen ions, which are found in acidic environments (Suthar et al., 2013). These nanoparticles consist of a corona and core layers (external and middle layers, respectively) that become soluble in acidic conditions, and release the substances contained within the core (Shen et al., 2008). This approach may allow for combined, localized delivery of chemotherapeutics with bicarbonate or basic buffers.

Implications of pH manipulation extend to clinical use of adoptive cell transfer therapy. Beyond increasing the ability of immune cells to migrate to the tumor site, reducing an acidic microenvironment can be used in a multi-stage treatment. Because neutralizing a tumor's extracellular pH has been shown to reduce metastasis in mice (Robey et al., 2009), administering a pH-neutralizing treatment may help decrease metastatic rates of a tumor. Doing so may improve the manageability of metastasized cells through ACT prior to administration.

Apart from ACT, extracellular pH neutralization can be used as a strategy to decrease chances of metastasis in early-diagnosis patients. Combination with chemotherapy may also be promising, and may be able to increase the efficacy of some classes of carbonic anhydrase inhibitors or proton pump inhibitors. Proton pump inhibitors can also be used to reduce acid production *in vivo* and neutralize the environment, a model that has been used to assess the effects of pH on ACT (Calcinotto et al., 2012). Increasing extracellular pH using carbonic anhydrases or proton pump inhibitors reduces the production of hydrogen ions in the tumor boundary (Neri & Supuran, 2011). Combination of these pH-manipulating drugs with a neutralized extracellular tumor environment would effectively accelerate the neutralization process of the drugs, which could increase their efficacy.

APPENDIX A: Buffer Toxicity

Introduction

Buffers used throughout each experiment were verified to ensure their efficacy through a preliminary buffer toxicity experiment. B-16 OVA were plated at acidic, neutral and basic conditions with MES, HEPES and Tricine, respectively. Initial pH measurements were measured at room temperature (25C) before being placed in the incubator at 37C. It was found that the pH of Tricine increased to 7.8, explaining the trend for cultures initially plated at 8.4 to decrease to 7.6. Thus, Tricine was not determined to be the most effective buffer for basic cultures.

An alternate buffer, TAPS, maintains a pH of 8.62 at 37C, making it a more suitable buffer. However, because TAPS is typically used for media with a pH value greater than 9, a second toxicity experiment was performed to determine if TAPS had a toxic effect on B16-OVA.

Methods

To determine whether TAPS had any more of a toxic effect than Tricine, two separate batches of media were made with the following recipes:

Unadjusted TAPS Allos Media	**Unadjusted Tricine Allos Media**
RPMI FBS Sodium Pyruvate Non-Essential Amino Acids (NEAA) L-Glutamine .042M TAPS B-mercaptoethanol	RPMI FBS Sodium Pyruvate Non-Essential Amino Acids (NEAA) L-Glutamine .058M Tricine B-mercaptoethanol

50,000 B16-OVA cells were plated in 12 different wells. 6 wells contained 1.5 mL of one TAPS, and the other 6 contained Tricine. Each well designated a time point for cell growth over 24 hours: the first well was counted after 24 hours, the second well counted after 48 hours, the third well after 72 hours, the fourth after 96 hours, the fifth after 120 hours, the sixth after 144 hours. Media was changed every 48 hours, with G418 put into each well both at the start of the experiment and when the media was changed.

Introduction

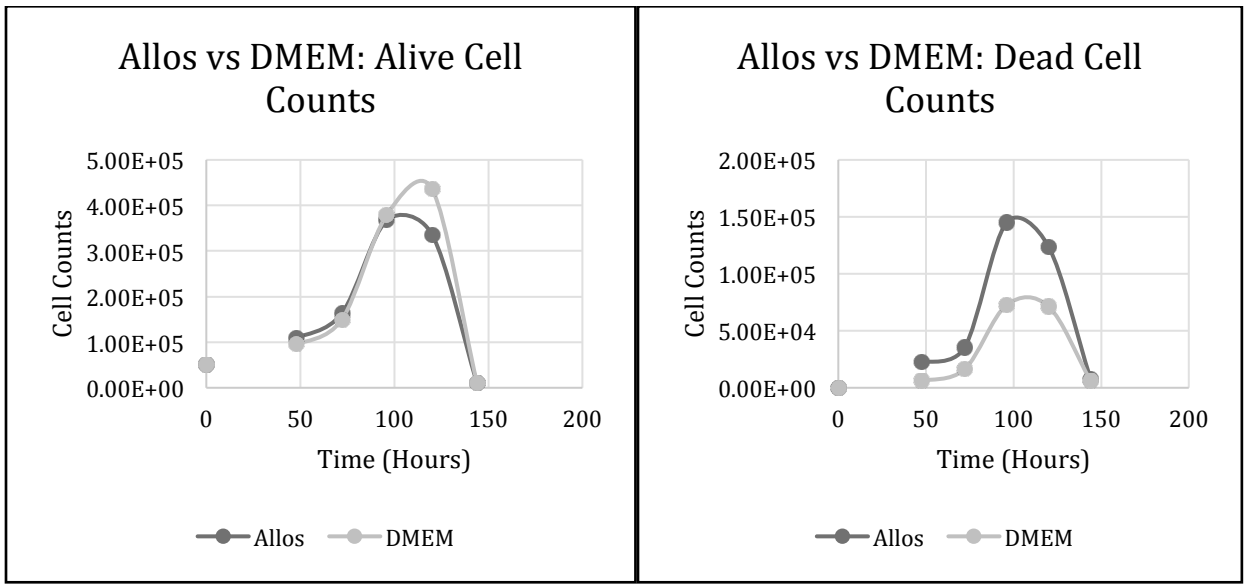
While initial trials with B-16 OVA were cultured with DMEM media, literature found B-16 OVA to have been successfully grown in Allos. It was hypothesized that eliminating the difference of media between Lymphoma and B-16 would allow future experiments to be more comparable. An experiment was performed to determine whether or not B16-OVA could grow sufficiently in Allos media. Lymphoma was not considered for this experiment because no literature provided evidence of EG-7 growth in DMEM.

Methods

Unadjusted DMEM Media	**Unadjusted Allos Media**
DMEM FBS	RPMI FBS Sodium Pyruvate NEAA L-Glutamine .058M HEPES B-mercaptoethanol

50,000 B16-OVA cells were plated in 12 different wells: 6 wells were cultured with 1.5 mL of DMEM and the other 6 were cultured with 1.5 mL of Allos. Each well designated a time point for cell growth over 24 hours: the first well was counted after 24 hours, the second well counted after 48 hours, the third well after 72 hours, the fourth after 96 hours, the fifth after 120 hours and the sixth after 144 hours. Media was changed every 48 hours, and G418 was put into each well both at the start of the experiment and whenever the media was changed.

Results



At the first time point, it was found that our counting procedure for concentrating the cells was in error, causing us to have unreliable cell counts. However, after the first time point, the error was resolved and we were able to continue the experiment, obtaining reliable trends from the latter 5 data points. Based on the curves above, there was little variance between the two media growth curves in the alive cell counts. However, the dead cell counts are discrepant, which may be due to user error. Overall, though, this experiment determined that Allos media performed sufficiently similarly to DMEM media, allowing it to be used in later experiments.

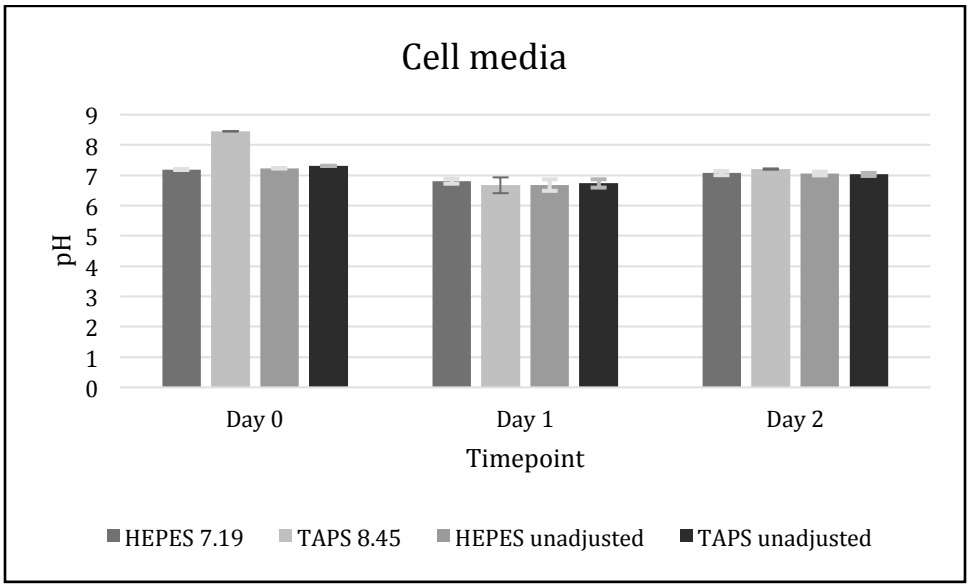
TAPS Buffer

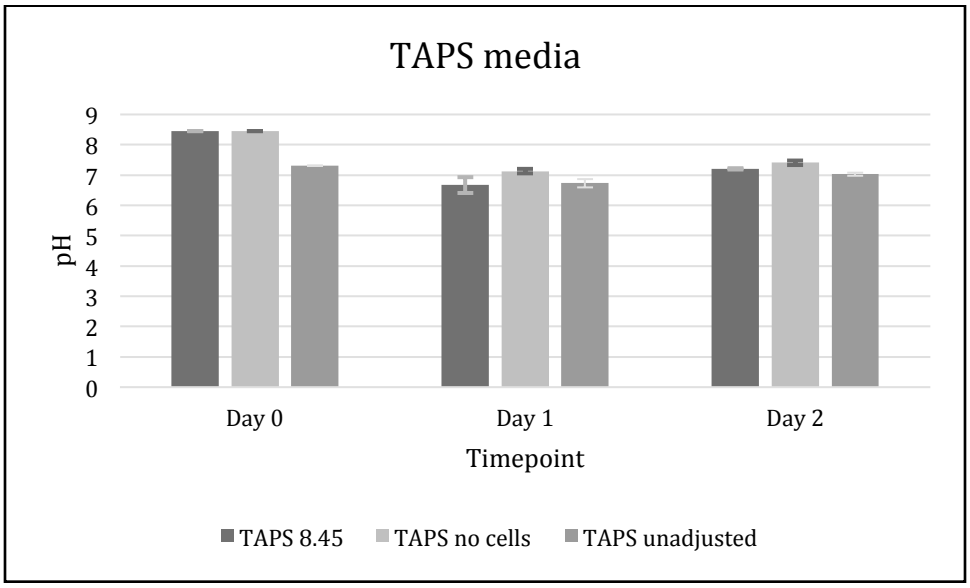
Unadjusted Regular Allos Media	**7.2 pH Regular Allos Media**	**Unadjusted TAPS Allos Media**	**8.4 pH TAPS Allos Media**
RPMI FBS Sodium Pyruvate NEAA L-Glutamine .0558M HEPES B-mercaptoethanol	RPMI FBS Sodium Pyruvate NEAA L-Glutamine .0558M HEPES** B-mercaptoethanol **Add NaOH or HCl as necessary to achieve the desired pH	RPMI FBS Sodium Pyruvate NEAA L-Glutamine .0411M TAPS B-mercaptoethanol	RPMI FBS Sodium Pyruvate NEAA L-Glutamine .0411M TAPS** B-mercaptoethanol **Add NaOH or HCl as necessary to achieve the desired pH

To determine whether TAPS could be used as a basic buffer, four different media groups were made as shown above, with three control groups of adjusted Allos with HEPES, unadjusted Allos with HEPES, and unadjusted Allos with TAPS. 50,000 cells were plated per well in this experiment, with 3 wells per media per each of the 6 24-hour time points. In total, there were 72 wells were plated, with 1.5 mL of respective media in each well. Therefore, 3 wells per media type were checked at 24 hours, 3 were checked at 48 hours, 3 were checked at 72 hours, 3 were checked at 96 hours, 3 were checked at 120 hours, and 3 were checked at 144 hours.

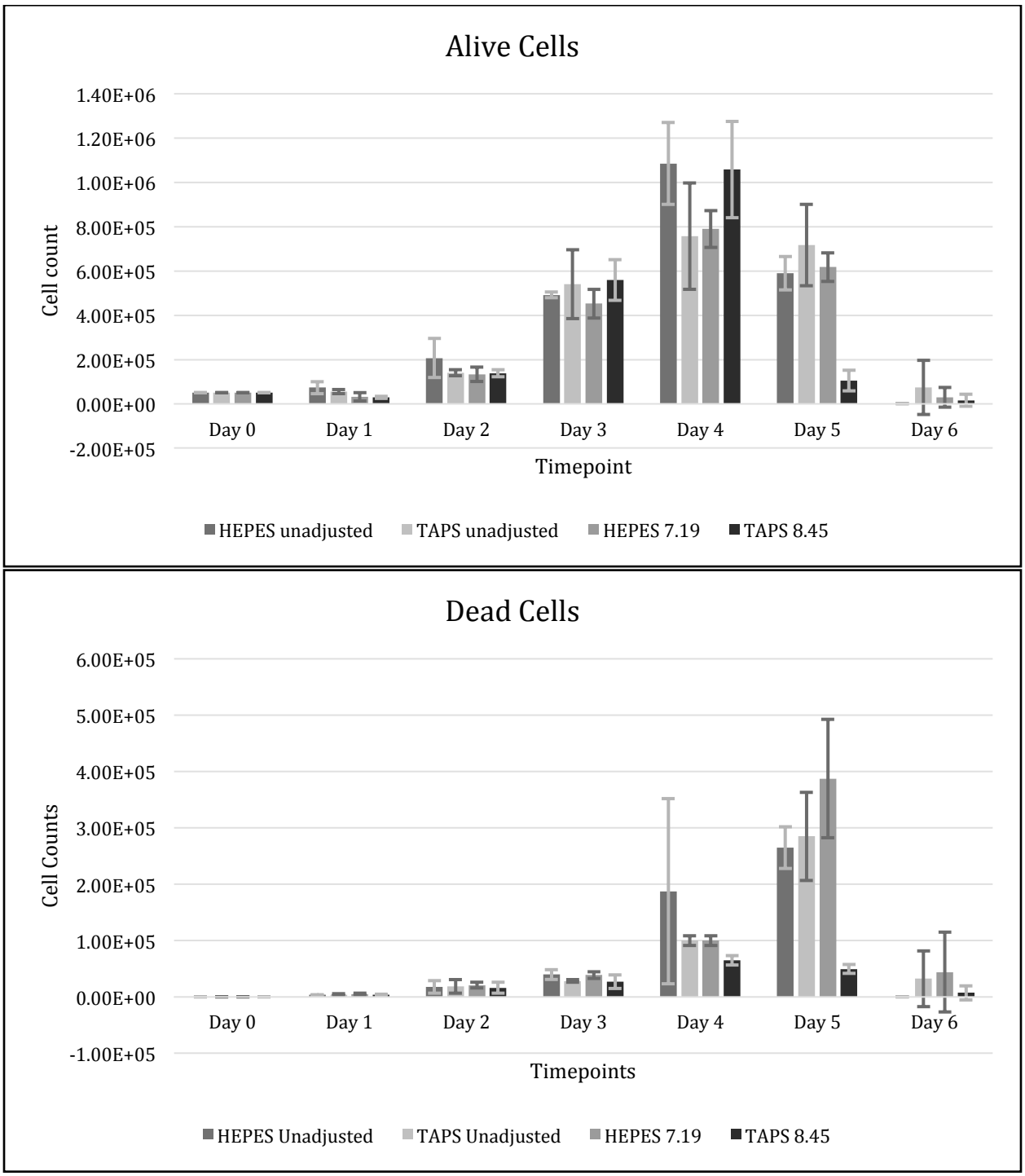
Additionally, the pH of the media was monitored for the first 48 hours at 24-hour time points to see how well TAPS maintained pH. For this latter part, TAPS media was placed in wells without any cells to serve as a control. Cells were observed under the microscope in their wells at each time point.

Results





Based on this data, Allos with TAPS buffer was not significantly different from the other media once placed in the incubator. It was unusual to find the pH increase in some cases as the cell growth progressed, but this increase in pH was not significant and may be secondary to pH probe user error. Traditional RPMI was also used in this experiment, meaning that the Bicarbonate concentration could have been a factor.



Overall, the microscope culture observations were consistent with the counts obtained, in that if the well appeared sparsely populated, the resultant cell counts were low. All media types changed from their initial color to an acidic yellow except for TAPS 8.4 when the media was

changed the second time at 120 hours. Before this media change cell counts for TAPS were not significantly different from the other media. Overall, TAPS proved to be no more effective at maintaining pH, or being lethal to the cells, than Tricine, and as Tricine was a more established buffer with this area of science, it was decided to use this buffer with further experiments.

APPENDIX C: Lab Equipment

pH Probe

A Mettler Toledo InLab pH combination electrode with XEROLYT polymer electrolyte was used to measure pH of our samples. The pH probe was calibrated before each trial. The pH probe was able to read pH ranges from 0 to 12 in temperatures from 0 to 100 Celsius. During storage, the pH probe was submerged in a potassium chloride solution to maintain the integrity of the electrode.

Microscope Camera

An attachable Motic Digital Microscope Moticam camera was used to take pictures of cells. The camera had up to 1.3 megapixels of live resolution, and included a 12mm focusable lens. Graphics of cells were taken at several magnitudes, including 4X, 10X, and 20X.

Luminometer

A Wallac 1640 luminometer machine was used quantitatively detect of light emitting markers. More specifically, the machine was used in Aim 3 to quantify relative light units in measuring relative Luminescence. The luminometer is suitable for a variety of settings including measuring glow luminometry, fluorometry and photometry.

APPENDIX D: Antibody Cocktails for Surface and Intracellular Staining

To perform surface staining, the following antibody cocktails were prepared:

Antibody Cocktail 1: PD1 PE, CD62L, CD127 APC, CD25 FitC

Antibody Cocktail 2: KLRG1 APC, CD44 PE, CD27 FitC

Single Staining Controls: CD25 PerCP, CD25 PE, CD25 PB, CD25 APC, and CD25 FitC

To perform intracellular staining, the following cocktails were prepared:

IFN-γ Antibody Cocktail: IFN-γ PE, TNFα APC

GZB Antibody Cocktail: GZB PE

Glossary

Activation- A three signal process that triggers proliferation, differentiation and cytokine secretion. This process must occur in order to generate cytotoxic T cells from naïve T cells.

Angiogenesis- Blood vessel formation. Tumor angiogenesis is the growth of new blood vessels that tumors need to grow.This process is caused by the release of chemicals by the tumor and by host cells near the tumor.

Antibody- A protein made by plasma cells (a type of white blood cell) in response to an antigen. Each antibody can bind to only one specific antigen.

Antigen- Any substance that causes the body to make an immune response against that substance. Antigens include toxins, chemicals, bacteria, viruses or other substances that come from outside the body.

Antigen presentation- A process in the body's immune system by which macrophages, dendritic cells and other cell types capture antigens, then enable their recognition by T-cells.

Assay- A laboratory test to find and measure the amount of a specific substance.

Biopsy- The removal of cells or tissues for examination by a pathologist.

Carcinogen- Any substance that causes cancer

CD8+ T Cell- The primary T cells involved in the destruction of the virus-infected or tumor cells.

CD4+ T Cell- Cells that enhance multiple cells in the immune system, including enhancing the growth of CD8+ T cells.

Chemoattractant- A chemical (chemotactic) agent that induces an organism or a cell to migrate toward it.

Chemokines- any group of low molecular weight cytokines identified on the basis of their ability to induce chemotaxis or chemokinesis in leukocytes (or in a particular populations of leukocytes) in inflammation.

Costimulation- The delivery of a second signal from an antigen-presenting cell to a T cell, which rescues an activated T cell from anergy, allowing it to produce the lymphokines necessary for production of additional T cells.

Cytokine- A type of protein that is made by certain immune and non-immune cells and has an effect on the immune system. Some cytokines stimulate the immune system and others slow it down. They can also be made in the laboratory and used to help the body fight cancer, infections and other diseases.

Cytostatic- A substance that slows or stops the growth of cells, including cancer cells, without killing them. These agents may cause tumors to stop growing and spreading without causing them to shrink in size.

Cytotoxic- Anything that kills cells, including cancer cells.

Cytotoxic T Cell (CTL)- An immune cell that is able to recognize only one type of antigen and kill any cell presenting that antigen.

Granzyme B- A protease that kills pathogens and cancer cells.

Hypoxic- Having too little oxygen.

Immunosuppression- Suppression of the body's immune system and its ability to fight infections and other diseases. Immunosuppression may be deliberately induced with drugs, as in preparation for bone marrow or other organ transplantation, to prevent rejection of the donor tissue.

In vitro- In the laboratory (outside the body).

In vivo- In the body.

Interferon Gamma (IFN-γ)- A cytokine that promotes the formation of additional CTLs.

Lyse- To cause or produce disintegration of a compound, substance, or cell.

Major Histocompatibility Complex (MHC)- A group of genes that code for proteins found on the surface of cells that help the immune system recognize foreign substances.

Malignant- Cancerous. Malignant cells can invade and destroy nearby tissue and spread to other parts of the body.

Metastasis- The spread of cancer from one part of the body to another.

Transgenic mice- Mice that have DNA from another source put into their DNA. The foreign DNA is put into the nucleus of a fertilized mouse egg. The new DNA becomes part of every cell and tissue of the mouse.

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